The Efficacy of Astaxanthin Supplementation on Indices of Exercise Recovery, Metabolism and Performance in Humans

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Abstract

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) is a liposoluble carotenoid usually ingested through the supplementation of *Haematococcus pluvialis*-derived antioxidant products. Based upon research conducted in mice, astaxanthin supplementation can improve indices of exercise recovery, metabolism and performance due to its potent antioxidant capacity. In exercising humans, similar observations have yet to be consistently realised. The following investigations were conducted to further explore the effect of astaxanthin supplementation on exercise recovery, substrate utilisation and endurance performance in recreationally active and trained human males. As a potent antioxidant compound, astaxanthin is suggested to provide a recovery benefit through the inhibition of pro-oxidant and pro-inflammatory intermediates post-exercise. This ability was investigated in Study 1 using a 30 min downhill run to induce muscle damage. In comparison to the placebo, indices of muscle damage and recovery post-exercise were not influenced by the prior eight-week intake of astaxanthin. Additionally, the lipophilic properties of astaxanthin allow it to protect and upregulate key metabolic enzymes involved in fat oxidation. Study 2 therefore utilised a graded exercise protocol to investigate whether four or eight weeks of astaxanthin supplementation could enhance the fat oxidative capacity during exercise. Again, no differences were reported when compared to the placebo. Astaxanthin has, however, been reported to peak in human plasma within the first week of intake. Study 3 was therefore the first to investigate and report an ergogenic and metabolic effect of astaxanthin following a shorter seven-day supplementation period, with improvements in performance and whole-body fat oxidation reported during a 40 km cycling time trial. Collectively, these studies highlight the importance of developing an optimal dosing strategy for astaxanthin intake based upon human pharmacokinetic data. With this knowledge, further explorations into the efficacy of astaxanthin in exercising humans can be made while using a supplementation strategy that has been developed from scientifically rigorous, evidence-based practice.

Declaration

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"The moment you doubt whether you can fly, you cease forever to be able to do it."

H.M Barrie, Peter Pan, constantly quoted by Karen and Garry Brown.

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4-HNE	4-hydroxy-2-nonenal
8-OHdG	8-hydroxy-2'-deoxyguanosine
ADI	Acceptable daily intake
AMPK	5' adenosine monophosphate-activated protein kinase
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
ARE	Antioxidant response element
AUC	Area under the curve
CA	Corrected absorbance
CAT	Catalase
CHox	Whole-body carbohydrate oxidation rates
CK	Creatine kinase
ConKE	Concentric knee extensors
CPT	Carnitine palmitoyltransferase
CRP	C-reactive protein
Cu	Copper
CV	Coefficient of Variation
DNPH	2,4-dintrophenylhydrazine
DOMS	Delayed onset of muscle soreness
EccKF	Eccentric knee flexors
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ETC	Electron transport chain
FAT/CD36	Fatty acid translocase/CD36
FAT_{max}	The exercise intensity at which fat oxidation is maximal
FAT_{min}	The exercise intensity at which fat oxidation becomes negligible
FATox	Whole-body fat oxidation rates
FAT_{zone}	The range of exercise intensities within 10% of the maximal fat oxidation rate
g	Hedges' *g*
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Glutathione disulphide
GXT	Graded incremental exercise test

H_2O_2	Hydrogen Peroxide
HR	Heart rate
HR_{max}	Maximal heart rate
ICC	Intraclass correlation coefficient
IKD	Isokinetic Dynamometer
IL-1β	Interleukin-1β
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MFO	Maximal fat oxidation rate
MFO_{FFM}	Maximal fat oxidation rate relative to fat free mass
MI	Mean improvement
Mn	Manganese
MPO	Myeloperoxidase
MVC	Maximal voluntary contraction strength
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
Nrf2	Nuclear factor erythroid 2-related factor
PC	Protein carbonyl
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PLA_2	Phospholipase A_2
$P\eta^2$	Partial eta squared
RER	Respiratory exchange ratio
ROF	Rating of fatigue
RPE	Rating of perceived exertion
RPE_L	Ratings of perceived exertion for the lower limb musculature
RPE_O	Ratings of perceived exertion for overall cardiovascular strain
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species

RNS	Reactive nitrogen species
SD	Standard deviation
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
TNF-α	Tumour necrosis factor-α
TT	Time trial
TTE	Time to exhaustion
VCO_2	Carbon dioxide excretion
VO_2	Oxygen consumption
$VO_{2max/peak}$	Maximal/peak oxygen consumption
W_{max}	Maximal power output
XO	Xanthine oxidase
Zn	Zinc

1. Introduction and Review of Literature

1.1. Overview

Reactive oxygen and nitrogen species (RONS) produced during exercise are recognised as fundamental stressors that can promote improvements in athletic performance and overall health (Busso, 2003, Radak et al., 2013, 2017). To ensure that improvements are sustained, recreationally active and trained individuals are required to manipulate the volume, intensity and frequency of each exercise bout over time (Busso, 2003). During periods of intense exercise training and competition, a system of interrelated endogenous antioxidant compounds (i.e. superoxide dismutase, catalase, glutathione peroxidase) work together to ensure RONS production does not become detrimental (Powers and Jackson, 2008). Multifaceted in its function, this system can prevent and delay the oxidation of biomolecules through removing, deactivating and preventing the formation of RONS (Bast and Haenen, 2015, Wagner, 2015).

If exercise becomes too vigorous, however, an excessive production of RONS can overwhelm the endogenous antioxidant defence system, causing a state of oxidative stress (Pingitore et al., 2015). Consequently, lipid, protein and nucleic molecules may become damaged, with potentially detrimental impacts on normal physiological function (Dalle-Donne et al., 2006, Peternelj and Coombes, 2011). The intake/ingestion of exogenous antioxidants has, therefore, become common practice in both recreationally active and trained populations (Peternelj and Coombes, 2011). As essential non-enzymatic molecules, exogenous antioxidants have the potential to supplement the endogenous antioxidant defence system if adequately sourced from the diet (Slattery, Bentley and Coutts, 2015). An example of a potent antioxidant that

can be sourced through either dietary intake or supplementation is the xanthophyll carotenoid, astaxanthin. Previously, astaxanthin has been suggested to enhance indices of exercise recovery, metabolism and performance as a result of its potent antioxidant capacity (Aoi et al., 2003, 2008, 2018, Ikeuchi et al., 2006, Earnest et al., 2011, Liu et al., 2014). Evidence to support this notion has been predominantly derived from studies using *in vitro* and *in vivo* animal models (Aoi et al., 2003, 2008, 2018, Ikeuchi et al., 2006, Liu et al., 2014), with similar observations in exercising humans yet to be consistently realised (Bloomer et al., 2005, Earnest et al., 2011, Djordjevic et al., 2012, Res et al., 2013, Baralic et al., 2015). The following chapter is written to 1) introduce the field of antioxidants and oxidative stress, and 2) critically appraise the current literature surrounding astaxanthin and its potential application as a dietary supplement within exercising humans. Underlying mechanistic factors pertaining to exercise recovery, metabolism and performance will also be considered alongside the identification of present and future areas of research focus.

1.2. Major Reactive Species

First detected in a biological system in 1954 (Commoner, Townsend and Pake, 1954), the term “free radical” is used to define an atom or a group of atoms that contain one or more unpaired electrons (Halliwell and Gutteridge, 2007). This imbalance in atom electron content often results in the production of a highly reactive and unstable molecule. As a collective, these reactive species are known as reactive oxygen or nitrogen species (RONS) (Neubauer and Yfanti, 2015). More commonly, RONS are

further categorised into either reactive oxygen species (ROS) or reactive nitrogen species (RNS).

The most abundant ROS is formed when oxygen is incompletely reduced, a biochemical reaction that yields the superoxide anion radical (Kerksick and Zuhl, 2015). Considered as relatively membrane impermeable, superoxide exhibits a moderately long half-life of ~ 5 s at a physiological pH (Marklund, 1976, Jaeschke, 2010) which enables it to diffuse and subsequently react with a number of intracellular targets (Powers et al., 2011). Despite this, superoxide is considered somewhat unreactive in comparison to other reactive species (Halliwell and Gutteridge, 2007). It does, however, possess an ability to react and produce a series of downstream derivatives, such as singlet oxygen, hydrogen peroxide (H_2O_2), hydroxyl radical intermediates and peroxynitrite, respectively (Reid, 2016a).

Singlet oxygen is a non-radical ROS since no electrons are unpaired and is formed during the dismutation of superoxide in water, or as a by-product of the reaction between peroxyl radical intermediates (Halliwell and Gutteridge, 2007, Powers et al., 2011). Although non-radical, the membrane permeability and no spin restriction of singlet oxygen allow it to possess a relatively high oxidising potential *in vivo* (Halliwell and Gutteridge, 2007, Powers and Jackson, 2008). Hydrogen peroxide is also a non-radical ROS, formed during the dismutation of superoxide by the superoxide dismutase enzymes (Halliwell and Gutteridge, 2007). Although H_2O_2 cannot oxidise lipid or nucleic biomolecules directly, it can diffuse within the cell as well as across the membrane, inactivating some enzymes, while also possessing cytotoxic properties that allow it to modulate certain inflammatory processes (i.e.

controlling cell proliferation and apoptosis) (Halliwell, Clement and Long, 2000, Powers and Jackson, 2008). Hydrogen peroxide can also be reduced further, forming the more potent and highly reactive hydroxyl radical intermediate (Kerksick and Zuhl, 2015). Although non-membrane permeable (Powers et al., 2011), the hydroxyl radical possesses a strong oxidising potential, making it capable of damaging protein, lipid and nucleic biomolecules (Dalle-Donne et al., 2006, Lipinski, 2011). Its high reactivity, however, leads to it exhibiting an extremely short half-life of ~ 1 ns *in vivo* (Kehrer and Klotz, 2015). As such, the detection of RONS are often dependent upon the measurement of downstream biomarkers most pertinent to the redox reactions taking place and/or the resultant damage to macro-molecular targets; including lipid, protein and nucleic biomolecules (Uttara et al., 2009, Powers et al., 2011, Margaritelis et al., 2016).

With superoxide considered as the parent molecule of the ROS cascade, nitric oxide (NO) is often considered as the parent molecule of the RNS cascade (Reid, 2016b). In healthy skeletal muscle, NO is predominantly synthesised via two NO synthases (NOS); neuronal NOS (nNOS) and endothelial NOS (eNOS) (Kobzik et al., 1994). A third NOS, inducible NOS (iNOS) is thought to not exist at significant levels in healthy skeletal muscle (Reid, 2016b), but is predominantly present in many inflammatory conditions (Powers and Jackson, 2008). Similar to superoxide, NO is thought to be neither highly toxic nor reactive *in vivo*, with its rapid removal enabled by its diffusion through tissues into red blood cells, where it is then converted to nitrate by reaction with oxyhaemoglobin (Pacher, Beckman and Liaudet, 2007). Nitric oxide can, however, react rapidly with superoxide, producing a more destructive and reactive peroxynitrite intermediate in the process (Halliwell, 1994, Pacher, Beckman and

Liaudet, 2007, Powers, Talbert and Adhihetty, 2011). Peroxynitrite is the destructive end point of the RNS cascade (Reid, 2016a), with its production reported to damage complex I of the electron transport chain (ETC), subsequently disrupting adenosine triphosphate synthesis (Riobó et al., 2001). It can also cause the oxidative damage of nucleic molecules, the nitration of proteins and the depletion of thiol groups (Powers et al., 2011). Paradoxically, it also indirectly alters cellular pathway signalling through reducing the bioavailability of both superoxide and NO, respectively (Reid, 2016b).

1.3. Sources of RONS

There are both exogenous and endogenous sources of RONS. Exogenous sources are characterised via an interaction with different environmental stressors; including exercise (Peternelj and Coombes, 2011). Understanding the interaction between exercise and endogenous RONS production is key when elucidating the potential efficacy of antioxidant supplementation. Across the exercising skeletal muscle, various sources of RONS exist, including; the mitochondria, sarcoplasmic reticulum, transverse tubules, sarcolemma and cytosol, with enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO), NOS and phospholipase A_2 (PLA_2) also associated (Powers and Jackson, 2008, Powers, Talbert and Adhihetty, 2011).

Originally, complexes I and III of the mitochondrial ETC were thought to be the main sources of RONS production within the exercising skeletal muscle (Davies et al., 1982), with early research quantifying this as 2-5% of total mitochondrial respiration during exercise (Boveris and Chance, 1973, Loschen et al., 1974). More recent data,

however, does not support this estimate, indicating that mitochondrial respiration actually contributes a modest 0.15% towards RONS production (St-Pierre et al., 2002). Higher levels of mitochondrial RONS production are also reported during basal respiration when compared to any form of active respiration (Di Meo and Venditti, 2001). It is therefore clear that additional sources need to be considered, especially during exercise.

The NADPH oxidase enzymes are now considered as predominant sources of intracellular RONS production (Sakellariou et al., 2013, Jackson, Vasilaki and McArdle, 2016), with XO and NADPH oxidase enzymes also thought to contribute to superoxide release into the extracellular space. Localised to the sarcoplasmic reticulum, transverse tubules and sarcolemma (Powers et al., 2011, Jackson, Vasilaki and McArdle, 2016), NADPH oxidases are released during the depolarisation of contracting skeletal muscle. Once released, these enzymes catalyse the transfer of electrons from NADPH to molecular oxygen, yielding superoxide (Hidalgo et al., 2006, Sakellariou et al., 2013). The XO pathway, in comparison, is triggered under conditions of intense skeletal muscle contraction, hypoxia or ischemia, whereby XO utilises molecular oxygen when converting hypoxanthine into xanthine and uric acid, again yielding superoxide (Harris, Sanders and Massey, 1999, Kerksick and Zuhl, 2015). Although their specific roles during exercise require further elucidation, NADPH oxidase activity is reported to be upregulated by endurance exercise (Dong et al., 2011), with XO associated with eccentric exercise and the secondary inflammatory response (Hellsten et al., 1997). As such, the NADPH oxidase and XO enzymes are now considered as key contributors to the whole-body oxidative response to exercise (Jackson, Vasilaki and McArdle, 2016).

Additional sources of RONS include the NOS and PLA_2 enzymes. In healthy skeletal muscle, nNOS and eNOS are considered the two major sources of NO, with iNOS associated with many inflammatory conditions (Kobzik et al., 1994, Reid, 2016b). The NOS enzymes produce NO continuously during the synthesis of L-arginine (Powers and Jackson, 2008), however, this production is increased further during the contraction of skeletal muscle (Kobzik et al., 1994). Endothelial NOS is localised to the muscle mitochondria (Kobzik et al., 1995, Powers and Jackson, 2008), with the NO produced at this site reported to bind to complex IV of the ETC, producing superoxide via the oxidation of ubiquinol, an upstream electron carrier (Poderoso et al., 1996, Riobó et al., 2001). In comparison, nNOS is localised to the muscle sarcolemma, where it is strongly expressed by fast-twitch muscle fibres and appears to be the primary source of NO within the skeletal muscle during exercise (Hirschfield et al., 2000, Powers et al., 2011). The PLA_2 enzymes, in contrast, stimulate intracellular RONS production in the mitochondria and cytosol, as well as the release of RONS into the extracellular space via lipoxygenase-dependent systems (Powers, Nelson and Hudson, 2011). While calcium-independent PLA_2 is suggested to be a major determinant of RONS activity under basal conditions, calcium-dependent PLA_2 is activated when intracellular calcium levels are elevated, implicating its role in RONS production within the contracting skeletal muscle (Powers, Nelson and Hudson, 2011, Jackson, Vasilaki and McArdle, 2016).

1.4. The Antioxidant Defence System

Free radicals can become reduced, a redox reaction that involves the gaining of an electron from a target molecule causing said molecule to become oxidised (Dröge, 2002, Slattery, Bentley and Coutts, 2015). Although this process of electron transfer can initiate adaptive and regulatory cellular signalling cascades (Powers et al., 2010), uncontrolled oxidation of target molecules can become maladaptive, initiating a state of oxidative stress (Alessio et al., 2000). As such, there exists a plethora of interrelated antioxidant compounds, that in relatively low concentrations, work synergistically to prevent or delay the oxidation of biomolecules (Bast and Haenen, 2015). Forming the antioxidant defence system, these multifunctional compounds can; donate electrons to highly reactive species, reducing them to less active derivatives, prevent less reactive molecules from becoming more potent, limit the availability of pro-oxidant materials, and even aid in the repair of RONS-induced damage (Powers et al., 2011, Slattery, Bentley and Coutts, 2015, Wagner, 2015).

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) are often considered as the primary enzymatic endogenous antioxidants, with glutathione and uric acid considered as the primary non-enzymatic antioxidants (Powers and Jackson, 2008, Peternelj and Coombes, 2011). First discovered in 1969 (McCord and Fridovich, 1969), SOD catalyses the dismutation of superoxide, yielding the less reactive H_2O_2 alongside molecular oxygen (Halliwell and Gutteridge, 2007). To facilitate this function, SOD associates with transition metals such as copper (Cu), zinc (Zn) and manganese (Mn) to form CuZn-SOD and Mn-SOD (Culotta, Yang and O'Halloran, 2006). In the intracellular space CuZn-SOD is

localised to the cytosol and the mitochondrial intermembrane space and is often termed SOD1, with CuZn-SOD in the extracellular space often termed SOD3 (Powers et al., 2011). In contrast, the Mn-SOD isoform, often termed SOD2, is localised to the intracellular compartment, inside the mitochondrial matrix (Kerksick and Zuhl, 2015).

The ubiquitous enzyme CAT also associates with a transient metal, in this case iron, and is responsible for the transformation of H_2O_2 into water and molecular oxygen (Powers et al., 2004). Similar to CAT, GPX is also responsible for the scavenging of H_2O_2 (Holmgren et al., 2005). Localised to the cytosol and mitochondria, GPX holds a greater affinity to H_2O_2 and therefore exerts a greater antioxidant effect (Tsutsui, Kinugawa and Matsushima, 2011). Its function, however, is dependent upon glutathione, a non-enzymatic antioxidant which recycles oxidised GPX back to a reduced/functional state (Powers et al., 2004). Glutathione achieves this through donating an electron to GPX, a process which oxidises glutathione, forming glutathione disulphide (GSSG). Due to its functional importance, the reduction of GSSG back to glutathione is paramount, a process accomplished by the GR enzyme, with NADPH acting as the reducing agent (Powers and Jackson, 2008).

Glutathione, therefore, is a clear example of the intercalation that exists between enzymatic and non-enzymatic antioxidant defences. In addition to reducing GPX, glutathione can also reduce and recycle pro-oxidant forms of vitamins C and E (Halliwell and Gutteridge, 2007, Villanueva and Kross, 2012), as well as reacting directly with, and reducing, a number of RONS (Yu, 1994). Another non-enzymatic antioxidant that functions as an electron donor is uric acid (Halliwell and Gutteridge, 2007). Produced during the conversion of hypoxanthine into xanthine by XO, uric acid

associates with iron and copper, and subsequently prevents the production of hydroxyl radical intermediates (Halliwell and Gutteridge, 2007, Powers et al., 2011).

As SOD, CAT and GPX are associated with the mitochondria, it is perhaps unsurprising that these enzymes are all prominent within oxidative type I muscle fibres (Powers et al. 1994, Powers et al. 2004). Furthermore, endurance exercise is reported to upregulate both SOD and GPX activity within the skeletal muscle (Criswell et al., 1993, Powers et al., 1994), with evidence regarding CAT currently less clear (Powers and Jackson, 2008). Glutathione is also reported as being upregulated in skeletal muscle fibres following endurance exercise training (Sen et al., 1992, Leeuwenburgh et al., 1997, Ohkuwa, Sato and Naoi, 1997). Although this finding is not currently replicated consistently for uric acid (Powers et al., 2011), it is accepted that prolonged endurance exercise can cause its upregulation, making it plausible that it could exert some form of antioxidant function during exercise (Neubauer et al., 2008).

Evidence therefore suggests that adherence to endurance exercise training provides a fundamental stressor, that if controlled, can upregulate many components of the endogenous antioxidant defence system. As such, the efficacy of exogenous antioxidant supplementation has been debated, with previous research questioning whether an overprotection from RONS could attenuate the exercise-induced adaptive responses to this fundamental stressor (Gomez-Cabrera et al., 2008, Ristow et al., 2009). If exercise training becomes too vigorous, however, this same stressor can become deleterious, overwhelming the endogenous antioxidant defence system, causing a state of oxidative stress (Pingitore et al., 2015). As such, the intake/ingestion of exogenous antioxidants has become common practice in both recreationally active

and trained populations (Peternelj and Coombes, 2011). As essential non-enzymatic molecules, exogenous antioxidants have the potential to supplement the endogenous antioxidant defence system if adequately sourced from the diet (Slattery, Bentley and Coutts, 2015). The following section is written to introduce and critically appraise the current literature surrounding a novel and potentially potent antioxidant named astaxanthin and its potential application as an exogenous antioxidant supplement within exercising humans. Underlying mechanistic factors pertaining to exercise recovery, metabolism and performance will also be considered alongside the identification of present and future areas of research focus.

1.5. Introduction to Astaxanthin

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) is a naturally occurring carotenoid found in marine species, such as microalgae, crustacea, fish and some birds (Guerin, Huntley and Olaizola, 2003, Hussein et al., 2006). Utilised in aquaculture, astaxanthin provides the characteristic reddish pigment to farm-raised salmon tissue (Ambati et al., 2014). Following the initial work of Kurashige et al. (1990) and Miki (1991), however, an alternative use for astaxanthin as a potent antioxidant compound in both *in vitro* and *in vivo* systems has been suggested. As it is oxygenated ($C_{40}H_{52}O_4$), astaxanthin is classified as part of the xanthophyll sub-species of the carotenoid family (Visioli and Artaria, 2017), with its potency seemingly underpinned by its structure at a molecular level (Miki, 1991, Goto et al., 2001, Guerin, Huntley and Olaizola, 2003, Hussein et al., 2006). With a molecular mass of 596.84 $g \cdot mol^{-1}$, astaxanthin contains two β-ionone ring systems within its structure that are linked by

a polyene chain and contain the oxygenated keto and hydroxyl moieties (Visioli and Artaria, 2017) (Figure 1.1). The presence of the polyene chain alongside each moiety enables astaxanthin to exert multiple antioxidant functions, namely in the scavenging and quenching of RONS, both within and at the surface of the phospholipid membrane (Miki, 1991, Goto et al., 2001) (Figure 1.2).

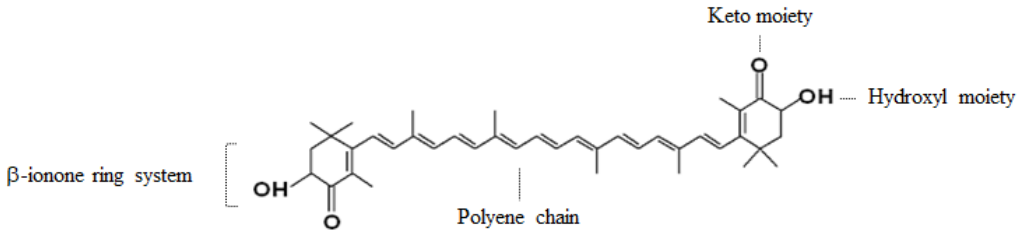

Figure 1.1 The chemical structure of astaxanthin.

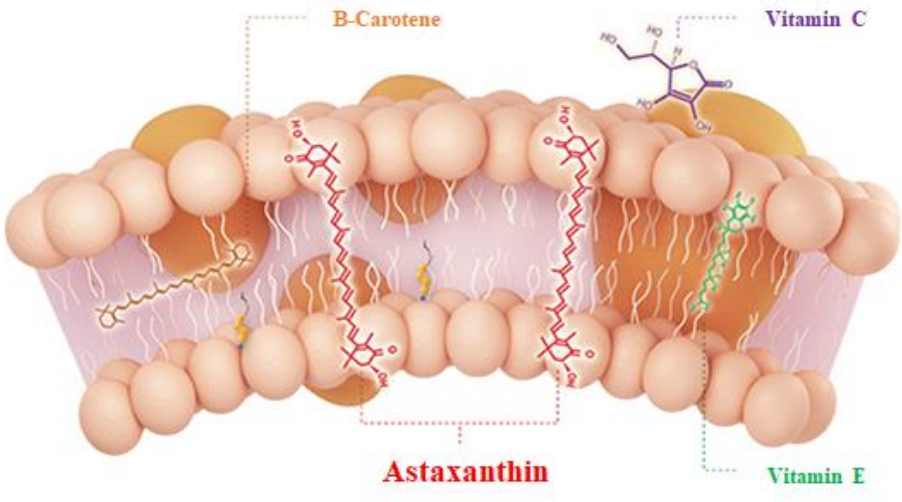

Figure 1.2 Schematic depicting the ability of astaxanthin, in comparison to other popular phytochemicals, to be able to exert its antioxidant function both within and at the surface of phospholipid membrane (AstaReal®, 2019).

1.6. Sources of Astaxanthin

As the human organism is unable to synthesise astaxanthin naturally, its uptake is dependent upon the dietary intake of foods, such as salmon, lobster, shrimp and crab (Visioli and Artaria, 2017). Whilst current data regarding the dietary intake of astaxanthin are limited, it is estimated that more than 70% of salmon production worldwide is farm-raised where astaxanthin is utilised as a feeding additive (Visioli and Artaria, 2017). If it is assumed that all dietary sources of seafood are obtained from aquaculture, it is estimated that the average European adult would consume between 0.8 and 2.0 $mg \cdot day^{-1}$ astaxanthin, with the higher percentile not expected to exceed intakes of 4.1 $mg \cdot day^{-1}$ (EFSA FEEDAP Panel, 2005). Alternatively, the intake of astaxanthin is possible through regular dietary supplementation, with a typical intake of 4 $mg \cdot day^{-1}$ astaxanthin recommended across several brands of commercially produced astaxanthin products (EFSA NDA Panel, 2014). Existing as three separate stereoisomers [(3S, 3'S), (3R, 3'R), and (3R, 3'S)] (Shah et al., 2016), the commercial applications of astaxanthin range from daily capsules and soft gels to energy drinks and powders (Ambati et al., 2014). Although the production of astaxanthin for aquacultural purposes can be sourced from a range of synthetic and natural sources, the only form currently commissioned for direct human consumption as a commercial product is the (3S, 3'S)-isomer (Shah et al., 2016, Visioli and Artaria, 2017). As a result, recent endeavours have been made to improve the production of astaxanthin from natural sources, such as *Haematococcus pluvialis*, to ensure that demands for human applications can be achieved (Ambati et al., 2014).

1.7. Bioavailability of Astaxanthin in Humans

Upon ingestion, it is assumed that the intestinal absorption of carotenoids, such as astaxanthin, are similar to those of dietary lipids (Visioli and Artaria, 2017). Carotenoids, for example, associate with lipid micelles in the lumen following consumption, a process which allows for passive diffusion into the lining of the intestinal mucosa. Following this, carotenoids then associate with chylomicrons and are subsequently released into the lymphatic system. At the liver, chylomicrons are digested by lipoprotein lipase, a process which allows carotenoids to assimilate with other lipoproteins, such as low-density lipoproteins, to be subsequently transported to other tissues in the body; including both adipose and muscle tissue (Visioli and Artaria, 2017).

Bioavailability data is available from research that has quantified the uptake and elimination kinetics of astaxanthin in human plasma using high-performance liquid chromatography (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Mercke Odeberg et al., 2003, Coral-Hinostroza et al., 2004, Rüfer et al., 2008). This method of analysis was used by Rüfer et al. (2008) during the investigation of 28 healthy males over a 4 week period. Prior to study onset, astaxanthin concentrations were quantified as non-detectable, following which two randomised groups (n = 14 each group) consumed either 250 g of wild or aquacultured salmon daily, to obtain ~ 1.25 $mg \cdot day^{-1}$ astaxanthin (5 µg $astaxanthin \cdot g^{-1}$ salmon flesh). Following 6 days of consumption, astaxanthin concentrations reached a plateau of 33.7 ± 16.2 $nmol \cdot L^{-1}$ (wild salmon) and 52.4 ± 16.2 $nmol \cdot L^{-1}$ (aquacultured salmon), respectively. Concentrations did not significantly change for the remainder of the 4 week protocol

(Rüfer et al., 2008). It therefore appears that when the intake of astaxanthin is chronic, maximal concentrations can be achieved and maintained within the first week of intake, even when astaxanthin is obtained from different sources. This data, however, is collated from one study only, where astaxanthin was consumed as part of the diet, requiring a daily intake of 250 g salmon (Rüfer et al., 2008). Future research should, therefore, aim to clarify the bioavailability of astaxanthin from a variety of different sources, including supplementation, with information regarding the elimination kinetics also of importance to understand how the availability of astaxanthin may diminish in humans over time.

Currently, the European Food Safety Authority (EFSA) advise an acceptable daily intake (ADI) of 0.034 $mg \cdot kg^{-1} \cdot day^{-1}$ astaxanthin (2.38 $mg \cdot day^{-1}$ in a 70 kg human) (EFSA FEEDAP Panel, 2014, EFSA NDA Panel, 2014). Despite this, pharmacokinetic data are available from studies that have issued acute doses of 40 and 100 mg, respectively (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Mercke Odeberg et al., 2003, Coral-Hinostroza et al., 2004). Following the ingestion of an acute *H. pluvialis*-derived 40 mg capsule, maximal plasma concentrations of 55.2 ± 15.0 $\mu g \cdot L^{-1}$ astaxanthin were recorded in eight healthy male participants (Mercke Odeberg et al., 2003). In the same study, uptake was significantly enhanced if astaxanthin was ingested as one of three lipid-based formulations ($n = 8$ per group), with maximal plasma concentrations in the range of 90.1 and 191.5 $\mu g \cdot L^{-1}$ reported (Mercke Odeberg et al., 2003). As a result, it is advised that astaxanthin should be consumed alongside the intake of dietary fats to ensure uptake can be optimised (Okada, Ishikura and Maoka, 2009). In comparison, increased maximal concentrations of 1.3 ± 0.1

$mg \cdot L^{-1}$ and 0.28 ± 0.12 $mg \cdot L^{-1}$ are reported in the plasma following an acute intake of 100 mg astaxanthin (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Coral-Hinostroza et al., 2004). Although a high level of variance exists between the two studies, this may be explained by the low sample sizes (n = 3 per study), which should be addressed in future research (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Coral-Hinostroza et al., 2004).

Additional information is also available regarding the pharmacokinetics of astaxanthin following acute supplementation regimes. Indeed, maximal blood astaxanthin concentrations are observed 8-10 h following the intake of 40 mg astaxanthin (n = 32) (Mercke Odeberg et al., 2003), with similar times of 6.7 ± 1.2 h (n = 3) and 11.5 h (n = 3) also observed following doses of 100 mg (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Coral-Hinostroza et al., 2004). Furthermore, a half-life of 15.9 ± 5.3 h is reported following a 40 mg dose (Mercke Odeberg et al., 2003), with half-lives of 21 ± 11 and 52 ± 40 h reported following the intake of a 100 mg dose (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Coral-Hinostroza et al., 2004). It therefore appears that the concentration–time profile of astaxanthin is monophasic following ingestion and can be described as a one-compartment model. Future research should seek to replicate these findings in doses equivalent to those advocated by the EFSA (0.034 $mg \cdot kg^{-1}$), as well as those available in commercial astaxanthin products (4 mg). In doing so, optimal dosing strategies may be developed and subsequently implemented for both chronic and acute methods of administration.

1.8. Mechanism of Action

In comparison to other popular phytochemicals, astaxanthin has previously been reported to possess a significantly greater antioxidant function (Kurashige et al., 1990, Miki, 1991, Shimidzu, Goto and Miki, 1996), with its antioxidant activities quantified as 10-fold greater than other carotenoids, such as β-carotene, and 100-fold greater than α-tocopherol (vitamin E) (Miki, 1991). In particular, astaxanthin seemingly holds an affinity for singlet oxygen and peroxyl radical intermediates (Miki, 1991, Shimidzu, Goto and Miki, 1996, Goto et al., 2001, Visioli and Artaria, 2017). Through the process of energy transfer, for example, astaxanthin is able to quench singlet oxygen, yielding ground state oxygen alongside astaxanthin in a triplet-excited state (Visioli and Artaria, 2017). As a carotenoid, astaxanthin is then able to dissipate this energy by interacting with surrounding solvent, returning back to the ground state structurally intact, ready to participate in further quenching cycles (Stahl and Sies, 2003, Visioli and Artaria, 2017). Additionally, astaxanthin is also able to scavenge and deactivate peroxyl radical intermediates, a function likely dependent upon the formation of resonance stabilised, carbon-centred radical adducts (Stahl and Sies, 2003, Visioli and Artaria, 2017). As such, an ability for astaxanthin to extensively protect lipid-rich structures against peroxidation during periods of oxidative stress has been suggested (Miki, 1991, Palozza and Krinsky, 1992, Naguib, 2000, Goto et al., 2001).

Research utilising animal models have also identified a potential for astaxanthin to indirectly modulate the endogenous antioxidant defence system through interacting with redox sensitive transcription factors, such as nuclear factor erythroid 2-related factor 2 (Nrf2) (Yang et al., 2011, 2014). At rest, Nrf2 is sequestered in the cytosol by

Kelch-like ECH-associated protein 1 (Keap1); however, during periods of oxidative stress, modifications to cysteine residues on Keap1 cause Nrf2 to be released and translocated to the nucleus where it binds to the antioxidant response element (ARE) (Kaspar, Niture and Jaiswal, 2009, Done and Traustadóttir, 2016). Once activated, the Nrf2–ARE signalling pathway initiates the transcription of several cytoprotective genes and enzymes capable of upregulating the endogenous antioxidant response to an oxidative stressor, potentially implicating Nrf2 in the beneficial effects of exercise (Done and Traustadóttir, 2016).

Similarly, phytochemicals can also stimulate the activation of the Nrf2–ARE pathway, a process which may occur through the modification of different cysteine residues to those targeted through exercise, suggesting a potential synergism between exercise and phytochemicals in the upregulation of antioxidant defence (Done and Traustadóttir, 2016). Although a specific mechanism of action has yet to be elucidated, research conducted in animal models report increases in Nrf2 expression, alongside the upregulation of endogenous antioxidant enzymes, including SOD, CAT and GPX, following astaxanthin administration (Yang et al., 2011, 2014, Xu et al., 2017). As key components of the endogenous antioxidant defence system (Powers et al., 2011), it is plausible that astaxanthin could activate endogenous antioxidant defence pathways upon administration. Future exploratory research should therefore be conducted to investigate this mechanism further, including the interaction between astaxanthin and Nrf2 in exercising humans.

1.9. Safety of Astaxanthin Supplementation

In 2014, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) advocated an ADI of 0.034 $mg \cdot kg^{-1} \cdot day^{-1}$ astaxanthin (2.38 $mg \cdot day^{-1}$ in a 70 kg human) based upon research previously conducted in rats (EFSA FEEDAP Panel, 2014). This was later reiterated by an EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), where it was concluded that the safety of 4 $mg \cdot day^{-1}$ astaxanthin (~ 0.06 $mg \cdot kg^{-1} \cdot day^{-1}$) had yet to be fully established (EFSA NDA Panel, 2014).

In contrast, no adverse effects were reported in blood pressure or biochemical parameters, including a metabolic panel and cell blood count, in 19 healthy participants supplementing with 6 $mg \cdot day^{-1}$ astaxanthin for 8 weeks (Spiller and Dewell, 2003). Similarly, no adverse effects were also reported in a recent exercise study where 16 trained individuals supplemented with 20 $mg \cdot day^{-1}$ astaxanthin for 4 weeks (Res et al., 2013). The acute intake of 40 mg astaxanthin has also been reported as "well-tolerated" in 32 healthy participants with only three mild events in the form of headaches reported in the 48 h post-intake (Mercke Odeberg et al., 2003). This was, however, in response to a single dose, with information concerning the chronic intake of 40 $mg \cdot day^{-1}$ not currently available in a healthy adult cohort.

Nevertheless, the chronic intake of 16 $mg \cdot day^{-1}$ and 40 $mg \cdot day^{-1}$ astaxanthin has been suggested as safe in patients suffering with functional dyspepsia (Kupcinskas et al., 2008). 131 patients were recruited and randomly assigned either 16 $mg \cdot day^{-1}$ astaxanthin (n = 43), 40 $mg \cdot day^{-1}$ astaxanthin (n = 44), or an appearance-matched control (n = 44) for 4 weeks. Although 36 adverse events were reported

during supplementation and a 4 week follow-up, prevalence was not significantly different between groups (16 mg·day^{-1}: 15 adverse events in 10 patients vs. 40 mg·day^{-1}: 8 adverse events in 7 patients vs. control: 13 adverse events in 7 patients) suggesting no additional safety issues (Kupcinskas et al., 2008). As a result, it may be possible to safely advocate both acute and chronic intakes of astaxanthin that are considerably greater than the current ADI (~ 2.38 mg·day^{-1}). Future research is therefore required to further elucidate the safety of astaxanthin so that human consumption guidelines can be adjusted accordingly.

1.10. Astaxanthin and Exercise Recovery

The completion of vigorous intensity exercise training sessions and competitive events are known to increase numerous physiological stressors, such as muscle damage, oxidative stress and inflammation (Leeder et al., 2012). Detriments to the skeletal muscle observed in response to the completion of vigorous intensity exercise may, therefore, not only result from damage directly induced by RONS, but also damage induced through the inflammatory cascade. If recovery is inadequate following exercise, it may prevent recreationally active and trained individuals from completing the subsequent exercise training sessions required to drive adaptation and/or performance improvements. Inadequate recovery may also increase risks of injury, illness and overtraining (Roberts et al., 2014). As a result, the investigation of strategies that can reduce the negative effect of exercise-induced muscle damage and/or accelerate the recovery process has become increasingly popular (Connolly,

Sayers and McHugh, 2003, Howatson and van Someren, 2008, Leeder et al., 2012, Hill et al., 2014).

Through its potency as an antioxidant compound, it is plausible to propose that astaxanthin could exert a recovery benefit through the inhibition of both pro-oxidant and pro-inflammatory intermediates (Aoi et al., 2003, Lee et al., 2003). *In vitro* research has reported a dose-dependent inhibition of NO and intracellular RONS, as well as the H_2O_2-induced activation of nuclear factor kappa B (NFκB) following 5–100 μM astaxanthin administration (Lee et al., 2003). Furthermore, 50 μM astaxanthin also completely inhibited the stimulation of iNOS and iNOS mRNA. As iNOS is predominantly present in many inflammatory conditions (Reid, 2016b), the subsequent expression of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), were also significantly suppressed (TNF-α: −76%; IL-1β: −46%) in comparison to the control (Lee et al., 2003).

Investigation with *in vivo* mouse models also report a similar effect following a lipopolysaccharide stressor, with significant reductions in plasma NO (−58%), TNF-α (−50%), and IL-1β (−57%) reported following treatment with a 40 $mg \cdot kg^{-1}$ dose of astaxanthin in comparison to the control (Lee et al., 2003). Although the work of Lee et al. (2003) was not specific to the recovery response to exercise, it displays a potential mechanism by which astaxanthin may promote recovery through inhibiting the oxidative and inflammatory responses to a physiological stressor. Supportive evidence is, however, received from an exercising mouse model (Aoi et al., 2003). Following a running TTE at an intensity of 28 $m \cdot min^{-1}$, 3 weeks of astaxanthin

supplementation (0.02 $w \cdot w^{-1}$ per 100 g of total dietary intake) attenuated oxidative damage to lipid and nucleic acid molecules in the skeletal and cardiac muscle tissue.

This was evidenced by the substantial decrease of 4-hydroxy-2-nonenal (4-HNE) modified protein production, as well as the significant inhibition of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the skeletal (−12%) and cardiac (−17%) muscle tissue, in comparison to the control (Aoi et al., 2003). Furthermore, 24 h post-exhaustive exercise, dietary astaxanthin also significantly decreased the activity of myeloperoxidase (MPO) by 50% in the skeletal muscle tissue and 33% in the cardiac muscle tissue when compared to the control, as well as significantly reducing creatine kinase (CK) activity in the plasma (astaxanthin: 2,617 $U \cdot L^{-1}$ vs. control: 3,032 $U \cdot L^{-1}$) (Aoi et al., 2003). As MPO and CK are markers of secondary neutrophil infiltration and muscle damage, this also highlights a potential for astaxanthin to attenuate the inflammatory muscle damage response in the days following exhaustive exercise.

The use of *in vitro* and *in vivo* mouse models have, therefore, demonstrated an ability of astaxanthin to inhibit a series of biomarkers linked to the onset of muscle damage, oxidative stress, and/or inflammation. As a result, research has subsequently aimed to elucidate whether astaxanthin supplementation possesses the ability to modulate the recovery response in an exercising human cohort. In a sample of 20 resistance trained males, for example, recovery from a muscle damaging bout of eccentric exercise was quantified through perceptions of muscle soreness, concentrations of plasma CK and the recovery of one repetition maximum concentric strength, mean isometric force, and mean dynamic force (Bloomer et al., 2005). Although a significant time effect for each variable was present in the 96 h post-exercise, a supplement effect of 4

mg·day^{-1} astaxanthin for three weeks prior to exercise was not observed. As young, resistance trained males were recruited, however, it is uncertain as to whether this outcome could be generalised to less trained individuals or to those participating in events other than high-force, resistance exercise.

Future research should, therefore, aim to address this uncertainty by recruiting participants with different training demographics to investigate whether training status can confound the efficacy of astaxanthin as a recovery aid post-exercise. More recently, markers of muscle damage and oxidative stress were measured in response to a 2 h bout of soccer exercise in elite youth male soccer players (n = 32; age: 17–18 years) following supplementation with 4 mg·day^{-1} astaxanthin for 90 days during the regular competitive season (Djordjevic et al., 2012). A supplement effect was not reported in exercise-induced changes in plasma CK and generic measures of oxidative stress (thiobarbituric acid-reactive substances (TBARS), advanced oxidation protein products, superoxide anion, total antioxidant status, sulphydril groups, and SOD), suggesting a recovery effect was not present (Djordjevic et al., 2012).

The same research group later conducted a similar investigation in a separate cohort of elite youth male soccer players (n = 40; age: 17–18 years) during the competitive season (Baralic et al., 2015). Adherence to exercise training alone over the 90 day protocol was reported to significantly attenuate muscle damage, implicated by a reduction in both plasma CK (astaxanthin: −44.6%; control: −30.1%) and lactate dehydrogenase (LDH) (astaxanthin: −27.4%; control: −18.5%) from baseline measures. Astaxanthin supplementation (4 mg·day^{-1}) was suggested to augment these reductions further, while also exerting a secondary anti-inflammatory effect through

attenuating training-induced increases in serum C-reactive protein and total leukocyte and neutrophil counts, in comparison to the control (Baralic et al., 2015).

Caution is required however, as a significant supplement effect of astaxanthin was not reported in regard to each of the aforementioned variables, apart from the LDH biomarker ($p < 0.05$); suggesting that astaxanthin actually had little effect. Furthermore, when attempting to elucidate the effect of astaxanthin on muscle damage and recovery responses to exercise, both Djordjevic et al. (2012) and Baralic et al. (2015) placed a heavy reliance upon indirect biomarkers such as CK and/or LDH obtained from the blood. Both CK and LDH have been reported to possess a large inter-individual variance and low reliability in blood concentrations (Connolly, Sayers and McHugh, 2003), with the use of muscular force production purported to be a more reliable marker of skeletal muscle injury (Warren, Lowe and Armstrong, 1999, Connolly, Sayers and McHugh, 2003). Future research should, therefore, be conducted with the use of more reliable analytical methods so that a comprehensive conclusion can be made regarding astaxanthin and the attenuation of exercise-induced muscle damage in exercising humans.

1.11. Astaxanthin and Exercise Metabolism

The metabolism of fat as an energy source is dependent upon the entry of long-chain fatty acids into the mitochondria; a process requiring the mitochondrial carnitine palmitoyltransferase (CPT) complex and in particular the CPT1 regulatory enzyme (Yeo et al., 2011). During exercise, RONS-induced oxidative damage to CPT1 can alter its function, attenuating the transportation of long-chain fatty acids and

consequently limiting the ability for fats to be oxidised as a viable energy source (Aoi et al., 2008). Due to its lipophilic properties, astaxanthin is known to accumulate in the mitochondrial membrane following consumption and provide a protection against RONS-induced detriments to its function (Wolf et al., 2010, Kidd, 2011). It is, therefore, hypothesised that through its function as an antioxidant, astaxanthin could protect CPT1 against RONS-induced oxidative modifications, causing an indirect enhancement of exercising fat metabolism in the process (Aoi et al., 2008).

Aoi et al. (2008) investigated this hypothesis with the use of an exercising mouse model. In comparison to the control, 4 weeks of astaxanthin supplementation (0.02% $w \cdot w^{-1}$) significantly enhanced the coimmunoprecipitation of fatty acid translocase (FAT)/CD36 and CPT1 by 14.5% due to a concomitant and significant 41.4% decrease in the oxidative modification of CPT1. Muscle glycogen concentrations were also significantly greater in the astaxanthin group (2.7 ± 0.1 $mg \cdot g^{-1}$ tissue) in comparison to the control (2.3 ± 0.1 $mg \cdot g^{-1}$ tissue) and plasma non-esterified fatty acid (NEFA) concentrations tended to be higher (astaxanthin group: 1.05 ± 0.05 $mEq \cdot L^{-1}$ vs. control group: 0.94 ± 0.05 $mEq \cdot L^{-1}$), suggesting a potential glycogen sparing effect (Aoi et al., 2008). In a separate cohort of mice undertaking the same supplementation protocol, the respiratory exchange ratio (RER) was significantly lower from 20 min onward during a 60 min run (25 $m \cdot min^{-1}$), with corresponding measures of fat oxidation ($mg \cdot kg^{-1} \cdot min^{-1}$) also significantly higher (+21%) and carbohydrate oxidation ($mg \cdot kg^{-1} \cdot min^{-1}$) significantly lower (-12%) when compared to the control (Aoi et al., 2008).

As a result, it was concluded that through its potency as an antioxidant compound, astaxanthin is able to indirectly enhance the utilisation of fats during exercise through increasing the intercalation of FAT/CD36 and CPT1 on the mitochondrial membrane (Aoi et al., 2008). Similar findings are also reported in a swimming mouse model (Ikeuchi et al., 2006). On completion of a 15 min swim against an additional 5% body mass, for example, mice supplemented with 6 and 30 $mg \cdot kg^{-1}$ astaxanthin for 3 weeks had significantly greater concentrations of plasma glucose (6 $mg \cdot kg^{-1}$: 124 $mg \cdot dL^{-1}$ vs. 30 $mg \cdot kg^{-1}$: 135 $mg \cdot dL^{-1}$ vs. control: 104 $mg \cdot dL^{-1}$), with plasma NEFA also significantly elevated throughout exercise in the 30 $mg \cdot kg^{-1}$ astaxanthin group. Muscle glycogen concentrations were also significantly greater post-exercise following 5 weeks of supplementation with 6 and 30 $mg \cdot kg^{-1}$ astaxanthin (6 $mg \cdot kg^{-1}$ group: 4.0 $mg \cdot g^{-1}$ vs. 30 $mg \cdot kg^{-1}$ group: 4.2 $mg \cdot g^{-1}$ vs. control group: 3.4 $mg \cdot g^{-1}$), with corresponding measures of liver glycogen concentrations also significantly greater in the 30 $mg \cdot kg^{-1}$ group (30 $mg \cdot kg^{-1}$: 49.8 $mg \cdot g^{-1}$ vs. control: 40.7 $mg \cdot g^{-1}$).

In contrast, a significant decrement in plasma NEFA concentration has been reported following the completion of a 30 min run at 25 $m \cdot min^{-1}$ in mice supplemented with 0.02 $w \cdot w^{-1}$ astaxanthin for 2 weeks (astaxanthin group: 905 ± 41 $mEq \cdot L^{-1}$ vs. control group: 1,151 ± 61 $mEq \cdot L^{-1}$) (Liu et al., 2014). Although the authors speculated that this resulted from increased utilisation within the skeletal muscle, no direct measurement was made (Liu et al., 2014). Furthermore, despite a tendency for plasma glucose to be higher post-exercise in the astaxanthin group (astaxanthin group: 121 ± 2 $mg \cdot dL^{-1}$ vs. control group: 112 ± 3 $mg \cdot dL^{-1}$), a significant supplement effect was

not reported, suggesting that astaxanthin did not exert a glycogen-sparing effect (Liu et al., 2014).

More recently, the metabolic mechanism of astaxanthin has been explored further in mice supplemented with *H. pluvialis*-derived astaxanthin (0.02 $w \cdot w^{-1}$) for 5 weeks (Aoi et al., 2018). Although indices of exercise metabolism were not measured in this study, levels of the regulatory enzyme 5' adenosine monophosphate-activated protein kinase (AMPK) were determined in the skeletal muscle post-supplementation through western-blotting. As a key regulator of skeletal muscle metabolism, AMPK is implicated in the stimulation of fatty acid oxidation through its control of malonyl-CoA and suppression of acetyl co-enzyme A carboxylase; the transportation of fatty acids into the mitochondria, potentially through FAT/CD36; as well as the upregulation of many transcription factors, such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), that are known to promote mitochondrial biogenesis and control mitochondrial oxidative capacity (Thomson and Winder, 2009). As mice in the astaxanthin group expressed a significantly greater level of skeletal muscle AMPK post-supplementation ($p < 0.05$), a potential mechanistic insight into how astaxanthin is able to enhance fat metabolism during exercise is provided (Aoi et al., 2018).

Evidence from exercising mice models would therefore suggest that 3–5 weeks of astaxanthin supplementation potentially exerts a metabolic benefit through enhancing fat utilisation and attenuating muscle glycogen depletion during endurance-type exercise (Ikeuchi et al., 2006, Aoi et al., 2008). It should, however, be noted that outcomes reported in an animal model are not always replicable in a human model

(Shanks, Greek and Greek, 2009). Interestingly, a similar metabolic effect has yet to be replicated within an exercising human cohort (Earnest et al., 2011, Res et al., 2013). Four weeks of 4 $mg \cdot day^{-1}$ astaxanthin supplementation, for example, did not influence parameters of substrate metabolism (such as RER, carbohydrate and fat oxidation rates, and plasma glucose and NEFA concentrations) obtained during 2 h of submaximal cycling (5% below lactate threshold) in trained male cyclists (n = 14; $VO_{2peak} \geq 50$ $mL.kg^{-1}.min^{-1}$) (Earnest et al., 2011).

The 4 $mg \cdot day^{-1}$ dose issued, however, was trivial in comparison to the 6–30 $mg \cdot kg^{-1}$ doses that have been previously prescribed in a mouse model (Ikeuchi et al., 2006). To elicit a similar metabolic modulation as previously reported in a mouse model, it was hypothesised that a dose greater than 4 $mg \cdot day^{-1}$ would be required in an exercising human cohort (Res et al., 2013). As a result, Res et al. (2013) recruited trained male cyclists or triathletes (VO_{2peak}: 60 ± 1 $mL \cdot kg^{-1} \cdot min^{-1}$) to a 4 week supplementation protocol consisting of either 20 $mg \cdot day^{-1}$ astaxanthin, or an appearance-matched control in a double-blind, parallel design (n = 16 astaxanthin group; n = 15 control group). Despite a fivefold increase in dose, measures of RER, carbohydrate and fat oxidation rates, and concentrations of plasma glucose and free fatty acids were again not significantly affected during the completion of a 1 h steady-state cycle at 50% of maximal power output (W_{max}) (Res et al., 2013). Based upon this evidence, it would therefore appear that 4 weeks of astaxanthin supplementation does not exert a metabolic effect during endurance exercise, even at greater levels of intake, in exercising humans.

Res et al. (2013) also detected no measurable changes in the plasma concentrations of the lipid peroxide malondialdehyde (MDA) during exercise, suggesting an antioxidant effect of astaxanthin was not present. Although speculative, this observation may provide an insight as to why a metabolic effect was not reported in this study, with previous research suggesting that an indirect metabolic modulation is mediated through the antioxidant protection of CPT1 from astaxanthin (Aoi et al., 2008). The lack of a direct antioxidant effect and the corresponding metabolic effect, reported by Res et al. (2013), may be attributable to either the high training and fitness demographics of the participants recruited (VO_{2peak}: 60 ± 1 $mL \cdot kg^{-1} \cdot min^{-1}$; training frequency: > 3 $sessions \cdot week^{-1}$ for ≥ 2 years) or the length of the supplementation period undertaken within this study (Res et al., 2013).

In trained individuals, for example, a reduction in RONS production is commonly reported at any given exercise intensity, with regular endurance-type exercise training also associated with decreased levels of lipid peroxidation due to an increased capacity of the endogenous antioxidant defence system (Radak et al., 2013). As such, Res et al. (2013) speculated that the improvements in endogenous antioxidant defence, induced by regular endurance-type exercise training, may attenuate the potential for additional improvements provided by astaxanthin supplementation. Concentrations of MDA, however, are reported to decrease significantly in sedentary individuals following 20 $mg \cdot day^{-1}$ astaxanthin for both 8 (astaxanthin group: 1.72 ± 0.28 $\mu mol \cdot L^{-1}$ vs. control group: 2.08 ± 0.12 $\mu mol \cdot L^{-1}$) and 12 weeks (astaxanthin group: 1.42 ± 0.29 $\mu mol \cdot L^{-1}$ vs. control group: 2.00 ± 0.24 $\mu mol \cdot L^{-1}$), respectively (Choi, Youn and Shin, 2011). Likewise, 12 weeks of 8 $mg.day^{-1}$ astaxanthin supplementation was also

reported to attenuate markers of lipid peroxidation in healthy untrained males (Karppi et al., 2007), suggesting that the demographics of participants may be a confounding variable for the antioxidant action, and therefore the corresponding metabolic effect, of astaxanthin.

Furthermore, the rate at which fat is oxidised during exercise is also subject to large inter-individual variations, even at the same absolute and relative intensities (Goedecke et al., 2000, Achten, Gleeson and Jeukendrup, 2002, Venables, Achten and Jeukendrup, 2005). The exercise intensity at which fat oxidation is maximal (FAT_{max}) is also highly variable between individuals (Goedecke et al., 2000, Venables, Achten and Jeukendrup, 2005). In 300 healthy men and women, for example, FAT_{max} values in the range of 25 and 77% VO_{2max} were reported despite an average value of 48 ± 1% VO_{2max} reported overall (Venables, Achten and Jeukendrup, 2005). Consequently, if fat oxidation is measured at a constant steady-state intensity, the ability to determine the true effect of a nutritional intervention may be diminished as some individuals will be exercising above and others below the exercise intensity at which FAT_{max} is elicited. Future research should consider participant demographics, the supplementation protocols administered, as well as incorporating sensitive measurements of exercising substrate utilisation. This may include employing an experimental protocol capable of detecting changes in metabolism across a range of exercise intensities (Achten, Gleeson and Jeukendrup, 2002, Venables, Achten and Jeukendrup, 2005), so that the metabolic effect of astaxanthin can be explored in more depth in exercising humans.

1.12. Astaxanthin and Exercise Performance

During endurance exercise, the depletion of muscle glycogen is commonly reported in the aetiology of fatigue; as such, methods aimed at attenuating this depletion, may provide an ergogenic benefit through delaying fatigue onset (Coyle et al., 1986). A metabolic mechanism that could potentially convey this benefit is the utilisation of fat as an alternative energy source to glycogen during exercise (Yeo et al., 2011). With previous research conducted in mice illustrating such a metabolic effect (Ikeuchi et al., 2006, Aoi et al., 2008), a potential for astaxanthin to act as an ergogenic aid during the performance of endurance exercise has been hypothesised (Ikeuchi et al., 2006, Aoi et al., 2008, Earnest et al., 2011, Res et al., 2013).

Ikeuchi et al. (2006) conducted a series of experiments in mice to investigate the ergogenic potential of astaxanthin (1.2, 6, or 30 $mg \cdot kg^{-1}$) on swim time to exhaustion (TTE). In the first experiment, mice undertook a weekly swim TTE against an additional 10% body mass over a 5 week supplementation period. In comparison to the control, a continuous significant improvement in TTE was observed from the first week onward in the mice supplemented with 6 $mg \cdot kg^{-1}$ ($p < 0.01$) and 30 $mg \cdot kg^{-1}$ ($p < 0.05$) astaxanthin. After 5 weeks, this significant improvement was observed in a dose-dependent manner across all astaxanthin groups with an ergogenic benefit also reported in the 1.2 $mg \cdot kg^{-1}$ astaxanthin group (1.2 $mg \cdot kg^{-1}$ group: 2.27 min vs. 6 $mg \cdot kg^{-1}$ group: 3.32 min vs. 30 $mg \cdot kg^{-1}$ group: 5.12 min vs. control group: 1.44 min) (Ikeuchi et al., 2006).

Similar results were also reported in a separate cohort of mice, as 3 weeks of 6 and 30 $mg \cdot kg^{-1}$ astaxanthin supplementation significantly improved swim TTE against an

additional 5% body mass (6 mg·kg^{-1} group: 27.50 ± 3.04 min vs. 30 mg·kg^{-1} group: 36.06 ± 4.13 min vs. control group: 19.45 ± 2.02 min) (Ikeuchi et al., 2006). Further support is also evident from running mice-models, as mice fed daily with 0.02 w·w^{-1} astaxanthin for 4 weeks were able to significantly enhance TTE at a running intensity of 30 m·min^{-1} by 34% (67.53 ± 4.20 min) in comparison to the exercising control (50.40 ± 5.00 min) (Aoi et al., 2008). A similar improvement in TTE at a running intensity of 25 m·min^{-1} was also reported in mice fed daily with *H. pluvialis*-derived astaxanthin (0.02 w·w^{-1}) for 5 weeks when compared to mice fed with either synthetic ($p = 0.032$) or *Phaffia* yeast-derived sources ($p = 0.015$) (Aoi et al., 2018). In comparison to the control, however, despite a 73% improvement in running TTE reported in the mice fed *H. pluvialis*-derived astaxanthin, this improvement did not reach statistical significance ($p = 0.106$, $n = 5$ each group); suggesting a supplement effect may not have been present (Aoi et al., 2018).

Based upon *in vivo* work conducted in mice, it therefore appears that 3-5 weeks of astaxanthin intake exerts an ergogenic benefit during the performance of endurance exercise; an ability attributed to its aforementioned effect on substrate metabolism (Ikeuchi et al., 2006, Aoi et al., 2008). In humans, a similar ergogenic benefit was reported in trained male cyclists ($n = 14$; $VO_{2peak} > 50$ mL·kg^{-1}.min^{-1}; weekly cycling volume > 160 km·week^{-1}), as 4 weeks of astaxanthin supplementation (4 mg·day^{-1}) significantly improved 20 km cycling time trial (TT) performance when compared to baseline (baseline: 39.47 ± 3.26 min vs. 4 weeks: 37.46 ± 3.10 min) (Earnest et al., 2011). Furthermore, the 121 s time improvement (5.1%) reported in the astaxanthin group was also significantly greater than the corresponding 18 s improvement (0.8%)

reported in the control (baseline: 37.31 ± 4.20 min vs. 4 weeks: 37.13 ± 4.43 min), suggesting a treatment effect was present ($p < 0.05$; effect size: 1.25) (Earnest et al., 2011).

In contrast, an ergogenic benefit was not reported during a 1 h cycling TT in trained male cyclists or triathletes ($n = 31$; VO_{2peak}: 60 ± 1 mL·kg^{-1}·min^{-1}; training frequency: > 3 sessions·week^{-1} for > 2 years) following a 4 week supplementation with either 20 mg·day^{-1} astaxanthin (baseline: 60.38 ± 1.20 min vs. 4 weeks: 59.14 ± 1.21 min) or an appearance-matched control (baseline: 59.43 ± 1.37 min vs. 4 weeks: 58.57 ± 1.39 min) (Res et al., 2013). Although there is no clear explanation for the disparity between the two findings, it should be noted that a metabolic mechanism was not reported by either study (Earnest et al., 2011, Res et al., 2013). As the enhancement of fat metabolism and the subsequent sparing of muscle glycogen are the suggested mechanisms by which astaxanthin exerts its ergogenic potential, the absence of this effect may implicate an alternative ergogenic mechanism in Earnest et al. (2011), or partially explain the absence of a performance effect in Res et al. (2013).

It is plausible, however, that the heterogeneity of the sample in Earnest et al. (2011) may have contributed to the disparity reported between studies with regards to TT performance. Participants ($n = 7$ each group) were randomly assigned to each treatment group and although a full familiarisation was completed, and baseline fitness characteristics appeared to be similar, the standard deviation (SD) of each 20 km TT (190–283 s) was far greater than the 136 s time improvement reported in the astaxanthin group. To ensure the reliability of the performance outcome reported, the re-test reliability of the 20 km TT should have been reported. As this information is

not available, it is difficult to conclude whether the time improvement reported in the astaxanthin group (5.1%) resulted from a significant supplement effect or the variation present in the study protocol. Previously, a coefficient of variation (CV) of 1.5% (± 10% confidence intervals: 1.1–2.1%) has been reported for the performance of a 20 km TT separated by 4 weeks in 19 trained cyclists (> 2 years racing experience at an A or B grade standard) (Clark et al., 2014). If a similar re-test reliability would have been reported by Earnest et al. (2011), the ergogenic effect of astaxanthin could be inferred as the performance improvement during the 20 km TT would have been greater than the CV reported. Future research should, therefore, seek to confirm whether an ergogenic potential of astaxanthin is present within an exercising human cohort, with a particular emphasis placed upon implementing a valid and reliable testing protocol to ensure that a robust conclusion can be made. In comparison to previous performance studies, future research should also consider implementing performance tests of a longer duration (>1 h), allowing the ergogenic potential of astaxanthin to be investigated during the completion of an exercise bout that may reflect the proposed mechanism of action more appropriately.

1.13. Summary and Research Aims

This review of literature has introduced three main research themes, namely the effect of astaxanthin on indices of exercise recovery, metabolism and performance. Together, these themes will inform the experimental investigations of this thesis. Previously, *in vitro* and *in vivo* animal models have provided evidence to support the use of astaxanthin as a dietary supplement in exercising humans. According to these

models, improvements in the acute recovery from exercise-induced muscle damage, substrate utilisation and endurance performance are reported following 3-5 weeks of astaxanthin intake; with each function attributed in part to the potent antioxidant capacity of this xanthophyll carotenoid. In exercising humans, however, these observations have yet to be consistently realised, with equivocal data reported. This thesis will attempt to address some of these issues with the following research aims and methodologies, written up as part of the proceeding three experimental chapters.

Study 1 (Chapter 3): Synonymous with downhill running exercise, muscular contractions that incorporate an eccentric component are often reported to cause significant microstructural damage due to repeatedly forcing the muscle to lengthen under tension (Proske and Allen, 2005). As such, the use of eccentric exercise protocols (such as downhill running) as a method of inducing muscle damage/soreness has become increasingly common in research aiming to investigate the recovery benefits of different nutritional interventions (Close et al., 2006, Mcleay et al., 2012, Hutchison et al., 2016). A 30 min downhill run, similar those conducted previously (Close et al., 2004, 2006), was used in Chapter 3 as an experimental protocol capable of inducing various markers of exercise-induced muscle damage and soreness so that the acute recovery function of astaxanthin could be investigated in a group of recreationally active male participants.

Therefore, the aim of Chapter 3 was to investigate whether a prolonged supplementation with astaxanthin (4 mg·day^{-1} and 12 mg·day^{-1}) for 8 weeks could improve the recovery of muscle function and attenuate various markers of muscle

damage/injury in the 48 h following a 30 min downhill run in recreationally active males.

Study 2 (Chapter 4): The FAT_{max} protocol was utilised in Chapter 4 as a previously validated, accurate and relatively reliable (CV: 9.6%) description of fat oxidation over a wide range of exercise intensities (Achten, Gleeson and Jeukendrup, 2002). This protocol, conducted on a treadmill, gradually increases the intensity (walking/running speed) of exercise until volitional exhaustion, with corresponding rates of fat oxidation quantified using indirect calorimetry. This ability to describe fat oxidation over a wide range of exercise intensities enables the determination of various parameters pertaining to the fat oxidative capacity; including the maximal fat oxidation rate (MFO) and the exercise intensity (% VO_{2peak} and % HR_{max}) at which this occurs (FAT_{max}). As such, the FAT_{max} protocol provided an experimental means to investigate the metabolic effect of astaxanthin on individual substrate utilisation across various exercise intensities and time points during supplementation.

As such, the aim of Chapter 4 was to investigate whether 4 weeks or 8 weeks of astaxanthin supplementation (4 $mg \cdot day^{-1}$ and 12 $mg \cdot day^{-1}$) could enhance the fat oxidative capacity, namely the MFO and FAT_{max}, during the completion of a FAT_{max} protocol in a group of recreationally active males.

Study 3 (Chapter 5): In Chapter 5, a 40 km cycling TT was chosen as an ecologically valid and reliable measure of endurance performance (Palmer et al.,

1996, Laursen, Shing and Jenkins, 2003, Currell and Jeukendrup, 2008). Indeed, previous research quantifies the test re-test reliability of the 40 km cycling TT as 1.0 ± 0.5% (Palmer et al., 1996) and 0.9 ± 0.7% following an initial familiarisation in trained male cyclists (Laursen, Shing and Jenkins, 2003). This high reproducibility, alongside the prolonged duration of the 40 km cycling TT (> 1 h), may provide a more appropriate experimental protocol to investigate the ergogenic potential of astaxanthin in exercising humans.

Therefore, the aim of Chapter 5 was to investigate whether a shorter 7 day intake of astaxanthin (12 mg·day^{-1}) could improve exercise performance and metabolism during the completion of a 40 km cycling TT in recreationally trained male cyclists.

2. General Methods

The purpose of the following chapter is to provide an outline of the general procedures that are consistent across all trials conducted in this thesis. This includes a description, justification and where necessary, the test re-test reliability of each procedure utilised. In Chapters 3-5, a more specific description of the methodologies and experimental designs employed are provided.

2.1. Ethical Considerations

Ethical approval was obtained for all studies in this thesis from the Department of Sport and Physical Activity Research Ethics Committee and the University Research Ethics Sub-Committee at Edge Hill University. If blood samples were collected and stored, this was practiced in accordance to the local Human Tissue Authority license regulations. Prior to study onset, all participants were first provided with a written information document detailing the procedures, benefits and risks of each study. Following a sufficient time to consider participation (usually 24 h), an opportunity to ask questions regarding the study was provided. If a participant agreed to volunteer, written informed consent was provided detailing this decision.

In accordance with Edge Hill University guidelines all participants completed a general health screening questionnaire prior to study onset to determine clearance to exercise. Participants with asthma were also required to present medication prior to exercise, with those advised against exercise by a doctor excluded. Participants also completed a health screening assessment before each exercise trial. This involved measuring resting blood pressure and heart rate (HR) with inclusion criteria set at $\leq$ 140 mmHg (systolic blood pressure), $\leq$ 90 mmHg (diastolic blood pressure) and $\leq$ 90

beats·min^{-1} (HR). If any measure exceeded this criteria exercise was not be permitted, with the participant advised to seek medical clearance to exercise. This was done to ensure all participants were able to safely carry out each exercise protocol without undue risk.

2.2. Participants

Healthy adult males between the ages of 18-45 years old were recruited to participate in each study. During participation, supplementation with additional forms of antioxidants/vitamins other than those provided in each study was not permitted. A list of commonly consumed astaxanthin-rich foods to avoid was also provided to limit the additional dietary intake of astaxanthin during supplementation.

Participant training status in each study was classified in accordance with the criteria outlined by De Pauw et al. (2013). As such, participants in Chapters 3 and 4 are referred to as recreationally active due to participating in a regular exercise regime that represents a volume of work that is $\geq$ 4 h·week^{-1}. In Chapter 5, participants are referred to as "recreationally trained cyclists", as although the VO_{2peak} (56.5 ± 5.5 mL·kg^{-1}·min^{-1}) and W_{max} (347 ± 38 W) of the sample met the criteria required to be classified as a "trained cyclist", the criteria required for total distance covered (60-290 km·week^{-1}) and cycling training frequency ($\geq$ 3 times·week^{-1}) was not met. As such, the term "recreationally trained cyclist" was deemed to be most appropriate for the participants recruited to Chapter 5 (De Pauw et al., 2013).

2.3. Pre-Experimental Procedures

Before each experimental trial participants undertook several standardised procedures to limit the confounding effects of nutrition, hydration status and day-to-day variations in the physiological response to exercise. Each experimental trial, for example, was conducted at a similar time of day (± 1 h) to account for the influence of circadian rhythm on exercise performance (Reilly, 1990). Participants were also instructed to refrain from strenuous exercise and the consumption of alcohol and caffeine in the 24 h preceding each visit (Rosenberg et al., 1978, Westerterp-Plantenga et al., 2006). A normal habitual diet was maintained in each study; however, participants were required to enter the laboratory in either a 4 h postprandial state (Chapters 3 and 5) or following an overnight fast (~ 10 h) (Chapter 4), except for the ingestion of water to ensure euhydration. Compliance with the above procedures was checked via 24 h dietary recall, with participants asked to replicate dietary intake prior to each trial.

2.4. Treatment

In Chapters 3 and 4 a parallel groups design was employed, with participants matched based upon characteristics pertinent to both performance capabilities (i.e. VO_{2peak}, FAT_{max}) and physical characteristics (i.e. age, body mass, body fat). Each matched group supplemented with one of three treatments for the entirety of the supplementation protocols (4 or 8 weeks). Treatment consisted of either 4 $mg \cdot day^{-1}$ astaxanthin, 12 $mg \cdot day^{-1}$ astaxanthin or an appearance-matched placebo with no viable constituents. In Chapter 5 a randomised, double-blind crossover design was employed. Participants supplemented with both 12 $mg \cdot day^{-1}$ astaxanthin and an

appearance-matched placebo with no viable constituents for the 7 days preceding each experimental trial. A 14 day washout period interspersed each supplementation period.

The use of a parallel groups design in Chapters 3 and 4 was required due to the implementation of a chronic supplementation protocol. It is accepted that this experimental design has less power than a randomised crossover design (as employed in Chapter 5) due to inter-individual variability (Burke and Peeling, 2018); however, this was somewhat controlled by the careful allocation of participants to each parallel group in each study as detailed above. In contrast, the shorter supplementation strategy implemented in Chapter 5 enabled the use of a randomised crossover design with each participant able to act as their own control. This is potentially advantageous, as it minimises the potential impact subtle differences in participant characteristics and individual responses to astaxanthin could have upon the outcome variable, increasing the statistical power of this study design (Cleophas and de Vogel, 1998, Burke and Peeling, 2018).

To reduce the potential of a carryover effect, whereby the treatment provided prior to the first experimental trial influences the outcome of the second experimental trial, the use of an appropriate washout period was paramount when conducting a randomised crossover design (Burke and Peeling, 2018). As such, the washout period undertaken in Chapter 5 was estimated based upon the calculations obtained from Saha (2018), which state that > 99.9% of a drug or treatment is eliminated after a period of time equivalent to 10 half-lives. Using the half-life of 15.9 ± 5.3 h, reported in Mercke Odeberg et al. (2003), it was estimated that > 99.9% of the total astaxanthin consumed would have been eliminated following a washout period of ~ 7 days. As this was an

estimation, however, a more conservative 14 day washout period was decided upon to ensure the elimination of astaxanthin prior to the second experimental trial.

The treatments administered in each study were produced and blister packaged by the pharmaceutical company AstaReal® (Sweden). Participants ingested two capsules daily (one morning and one evening), with compliance ensured via daily text message reminders and a pill count post-ingestion. Each treatment also had a randomised code assigned to it, ensuring that all studies were double-blind until after data analysis was complete. The 12 $mg \cdot day^{-1}$ dose administered in each experimental chapter was the maximal dose available to administer as stipulated by the manufacturer (AstaReal®, Sweden).

2.5. Environmental Conditions

All exercise trials in this thesis were performed in a temperate laboratory environment (temperature: 19 ± 2 °C; pressure: 753 ± 9 mmHg, humidity 45 ± 4%).

2.6. Anthropometric Measurements

On entering the laboratory height was measured using a stadiometer (Holtain, UK) and is subsequently reported in metres (m). Body mass (kg) and body fat (%) were measured using an air displacement plethysmography device (BodPod, Cosmed, Italy), with data reported to the nearest 0.1 kg/%. Prior to each measurement, calibration was carried out in accordance with the guidelines set by the manufacturer. This method of assessing body composition has been validated against other

techniques, such as dual-energy x-ray absorptiometry (Minderico et al., 2006), and is also reported to possess a high test re-test reliability for both within-day (reliability coefficient: 0.992-0.994) and between-days (reliability coefficient: 0.996) measurements obtained from healthy male participants (Tseh, Caputo and Keefer, 2010).

2.7. Heart Rate

To enable the continuous monitoring of heart rate participants wore a heart rate transmitter belt across their chest, level with the xiphoid process, which was subsequently connected to a telemetric monitoring system (Forerunner 15, Garmin, USA). Heart rate was recorded at several time points during each trial, with further descriptions provided in each study chapter.

2.8. Perceptual Variables

Ratings of perceived exertion (RPE) were assessed during exercise on a 6-20 point scale, with verbal descriptors provided to ensure that participants were able to select the appropriate rating (Borg, 1982). In Chapters 3 and 5, RPE was differentiated into ratings for overall cardiovascular strain (RPE_O) and for the lower limb musculature (RPE_L) to ensure that a rating of both central and localised fatigue/exertion could be obtained (Faulkner and Eston, 2007). This was particularly important in Chapter 5 as sensations of localised muscle fatigue in the legs have been reported to dominate the assessment of effort perception during cycling (Borg, Ljunggren and Ceci, 1985, Faulkner and Eston, 2007).

2.9. Gas Analysis

Breath-by-breath expired air was collected and analysed during each exercise trial with the use of an online gas analysis system (Oxycon Pro, Jaeger, Germany). The Oxycon Pro has previously been reported to be both valid and reliable, possessing a relatively low CV of 4.7-7.0% (Carter and Jeukendrup, 2002). Prior to each use, the system was calibrated in accordance with instructions set by the manufacturer. Gas analysers were calibrated using known gas concentrations (16.0% oxygen, 5.0% carbon dioxide), whilst volume calibration was conducted using a 3 L syringe (Hans Rudolph, USA) and the automated 2 L system setting. As data were collected continuously throughout exercise in Chapters 3 and 4, participants were required to wear a face mask (Hans Rudolph, USA) during exercise. In Chapter 5, as expired air was not collected continuously a face mask was not required, with a nose-clip and mouthpiece used when expired air samples were required at each data collection time point.

2.10. Determination of VO_{2peak}

All participants completed a graded incremental exercise test (GXT) to volitional exhaustion to enable the determination of peak oxygen uptake (VO_{2peak}) during exercise. In Chapters 3 and 4 the GXT was conducted on a motorised treadmill (H-P cosmos, Germany) and commenced at a speed of 3.5 $km \cdot h^{-1}$ and a gradient of 1.0%. Speed was then increased by 1.0 $km \cdot h^{-1}$ every 3 min until a RER of 1.00 was reached, at which point speed remained constant and the gradient was increased by a further 1.0% every min until a 3.0% gradient was achieved. If the participant had not yet

reached volitional exhaustion, the gradient was then kept constant and the speed was increased by 1.0 $km \cdot h^{-1}$ until volitional exhaustion was achieved. In Chapter 5 the GXT was conducted using an electromagnetically braked cycle ergometer (Lode Excalibur Sport, The Netherlands). Following 5 min of unloaded pedalling, the GXT commenced at 75 W and work rate was increased by 30 W every 1 min until volitional exhaustion. Participants selected a preferred cadence to maintain throughout, with volitional exhaustion defined as an inability to maintain $\geq$ 60% of this cadence for $\geq$ 5 s despite strong verbal encouragement. Breath-by-breath expired air was collected throughout each GXT for VO_{2peak} determination (OxyCon Pro, Germany). During analysis, data was averaged into 10 s intervals, with VO_{2peak} defined as the highest 30 s average of VO_2 obtained during the GXT.

2.11. Indirect Calorimetry

Indirect calorimetry is a method of determining whole-body substrate oxidation rates using measurements of oxygen consumption (VO_2) and carbon dioxide excretion (VCO_2). In Chapters 3 and 5, whole-body fat oxidation rates (FATox) were calculated using the VO_2 and VCO_2 data obtained during exercise, with whole-body carbohydrate oxidation rates (CHox) also calculated in Chapter 5. The following stoichiometric equations proposed by Jeukendrup and Wallis (2005) were used, assuming protein oxidation during exercise is negligible:

$$\textbf{FATox } (g \cdot min^{-1}) = 1.695 * VO_2 - 1.701 * VCO_2$$

$$\textbf{CHox } (g \cdot min^{-1}) = 4.210 * VCO_2 - 2.962 * VO_2$$

The respiratory exchange ratio (RER) was also calculated using the following equation as an indirect measure of substrate utilisation during exercise:

$$\mathbf{RER = VCO_2 / VO_2}$$

To completely oxidise 1 mol of glucose 134 L of oxygen is required and this produces 134 L of carbon dioxide. An RER of 1.00 is therefore indicative of glucose/ carbohydrate being the predominant fuel source (134 L/134 L = 1.00). For the complete oxidation of 1 mol of palmitic acid 515 L of oxygen is required and this produces 358 L of carbon dioxide. An RER of 0.70 is therefore indicative of palmitic acid/fat being the predominant fuel source (358 L/515 L = 0.70) (Jeukendrup and Wallis, 2005).

3. The Effect of Two Doses of Astaxanthin Supplementation on Indices of Exercise-Induced Muscle Damage and Recovery in Recreationally Active Males

3.1. Introduction

Muscular contractions that incorporate an eccentric component are often implicated in the aetiology of exercise-induced muscle damage due to repeatedly forcing the muscle to lengthen under tension (McHugh et al., 1999, Proske and Morgan, 2001). Synonymous with resistance training and downhill walking/running, detriments to muscular function and performance are often exhibited (Proske and Allen, 2005), with the concomitant production of RONS and inflammatory intermediates implicating further damage, delaying the recovery process and contributing to the delayed onset of muscle soreness (DOMS) (Close et al., 2004, Mcleay et al., 2012). If recovery is inadequate following exercise, it can prevent recreationally active and trained individuals from completing the subsequent exercise training sessions required to drive adaptation and/or performance improvements. Inadequate recovery can also increase risks of injury, illness and overtraining (Roberts et al., 2014). As a result, recovery strategies aimed toward alleviating symptoms of exercise-induced muscle damage and/or accelerating the recovery process continue to receive widespread research interest in sport nutrition and exercise physiology (Connolly, Sayers and McHugh, 2003, Howatson and van Someren, 2008, Leeder et al., 2012, Hill et al., 2014).

Through its potency as an antioxidant compound, it is plausible that astaxanthin could exert a recovery benefit through inhibiting both pro-oxidant and pro-inflammatory intermediates (Aoi et al., 2003, Lee et al., 2003). Evidence from both *in vitro* and *in vivo* mouse experimental models support this notion, demonstrating an ability for astaxanthin to inhibit a series of biomarkers linked to the onset of muscle damage,

oxidative stress, and/or inflammation (Aoi et al., 2003, Lee et al., 2003). In exercising humans these observations have yet to be realised, with a supplement effect of 4 $mg \cdot day^{-1}$ astaxanthin not reported in resistance trained males or elite youth male soccer players following 3 weeks and 90 days of supplementation, respectively (Bloomer et al., 2005, Djordjevic et al., 2012, Baralic et al., 2015). In Bloomer et al. (2005), however, the authors suggested that because resistance trained males were recruited, it is uncertain as to whether a similar no effect of astaxanthin would be reported in individuals unaccustomed to high-force resistance exercise. This is likely due to the repeated-bout effect, whereby regular adherence to muscle damaging bouts of resistance training may actually provide a protective adaptation, attenuating the muscle damage response to future exercise sessions (Proske and Allen, 2005). The current study, therefore, recruited recreationally active males unaccustomed to eccentric exercise and high-force resistance training, with the aim of investigating whether 4 $mg \cdot day^{-1}$ or an increased 12 $mg \cdot day^{-1}$ dose of astaxanthin for 8 weeks can attenuate the muscle damage response and/or accelerate the recovery process in the 48 h following a 30 min bout of eccentric exercise.

3.2. Methods

3.2.1. Participants

Twenty-five recreationally active males (age: 26 ± 5 years, height: 1.79 ± 0.05 m, body mass: 82.0 ± 9.1 kg, body fat: 17.1 ± 3.5%) provided written informed consent and participated in the study. All participants were unaccustomed to eccentric exercise/downhill running at study onset. Participants were not permitted to

supplement with additional antioxidants/vitamins alongside those provided in the current study. A list of commonly consumed astaxanthin-rich foods to avoid was also provided to limit the additional dietary intake of astaxanthin during supplementation. Participants were also instructed to refrain from strenuous exercise and the consumption of alcohol and caffeine in the 24 h preceding each visit (Rosenberg et al., 1978, Westerterp-Plantenga et al., 2006), and to enter the laboratory in a 4 h postprandial state, except for the ingestion of water to ensure euhydration.

Compliance with the above procedures was checked via 24 h dietary recall, with participants asked to replicate dietary intake prior to each trial. In addition, participants were also instructed to not undertake any form of therapeutic intervention used to alleviate symptoms of muscle soreness in the days leading up to and following the downhill run. All participants visited the laboratory at a similar time of day (± 1 h) on five separate occasions (two preliminary trials and three experimental trials). A double-blind parallel-groups design was employed.

3.2.2. Preliminary Trials

On entering the laboratory measures of height and body mass were obtained, with body composition also determined using air displacement plethysmography. Participants then completed a GXT to volitional exhaustion on a motorised treadmill. The GXT commenced at a speed of 3.5 $km \cdot h^{-1}$ and a gradient of 1.0%. Speed was then increased by 1.0 $km \cdot h^{-1}$ every 3 min until a RER of 1.00 was reached, at which point speed remained constant and the gradient was increased by a further 1.0% every min until a 3.0% gradient was achieved. If the participant had not yet reached volitional

exhaustion, the gradient was then kept constant and the speed was increased by 1.0 $km \cdot h^{-1}$ until volitional exhaustion. Breath-by-breath expired air was collected for VO_{2peak} determination and was defined as the highest 30 s average of VO_2 recorded during the GXT. Following a short period of rest, participants were then familiarised with the Isokinetic Dynamometer (IKD) protocol used during each experimental trial (as described in 3.2.5. Determination of Isokinetic Maximal Voluntary Contraction Strength). Participants completed this preliminary trial on two separate occasions, once at baseline and once more during the eighth week of supplementation. This was to ensure that each participant could be re-familiarised with the IKD protocol, but also to ensure that any changes in VO_{2peak} and/or isokinetic MVC could be highlighted before the first experimental trial.

3.2.3. Group Matching

Following the first preliminary trial participants were divided into three parallel groups, matched based upon the following characteristics; age (years), VO_{2peak} absolute ($L \cdot min^{-1}$) and relative ($mL \cdot kg^{-1} \cdot min^{-1}$), isokinetic maximal voluntary contraction strength (MVC) for the eccentric knee flexors (eccKF) (N·m) and the concentric knee extensors (conKE) (N·m), body mass (kg) and body fat (%).

Each group received one of three supplements, consisting of either 4 $mg \cdot day^{-1}$ astaxanthin (AstaReal®, Sweden) (n = 9), 12 $mg \cdot day^{-1}$ astaxanthin (AstaReal®, Sweden) (n = 8) or an appearance-matched placebo with no viable constituents (AstaReal®, Sweden) (n = 8). Participants ingested two capsules daily (one morning and one evening) for the entirety of the 8 week protocol, continuing until after the final

experimental trial had been completed. Compliance was ensured via daily text message reminders and a pill count post-ingestion. To ensure the study remained double-blind, each supplement was provided with a randomised alphanumerical code until after data analysis was complete.

3.2.4. Experimental Trials

Following the 8 week supplementation period, and within 7 days of the second preliminary trial, participants completed a 30 min downhill run on a motorised treadmill (H-P cosmos, Germany). Due to the repeated bout effect synonymous with eccentric-type exercise (McHugh, 2003, Proske and Allen, 2005), a familiarisation session was not completed. Participants were, however, familiarised with running on the motorised treadmill during the GXT, allowing for a degree of familiarity at trial onset. On entering the laboratory, a resting venous blood sample was obtained alongside pre-exercise measures of perceived muscle soreness and isokinetic MVC. After each of these measures were obtained, participants commenced with the downhill run. The run lasted for 30 min at a gradient of -12.0%, with speed ($km \cdot h^{-1}$) set to an intensity equivalent to 70% VO_{2peak} (13.3 ± 1.9 $km \cdot h^{-1}$). To verify this intensity breath-by-breath expired air was collected during the first 10 min of the downhill run, with speed altered until 70% VO_{2peak} was consistently achieved. The speed at 10 min then remained constant for the remainder of the protocol. Heart rate (HR) and RPE (Borg, 1982), for the whole-body (RPE_O) and the lower limbs (RPE_L) were measured at 5 min intervals during the run. Once completed, a post-exercise venous blood sample was obtained immediately, alongside the reassessment of

perceived muscle soreness and isokinetic MVC. Each of these measures were subsequently obtained 24 h post- and 48 h post-downhill run during two follow-up experimental visits.

3.2.5. Determination of Isokinetic Maximal Voluntary Contraction Strength

Participants were secured in a seated position on the IKD (Biodex Medical Systems, USA) at ~ 90° of hip flexion. Restraints were applied proximal to the knee joint across the thigh, waist and chest of each participant. The cuff of the lever arm was secured around the ankle, proximal to the malleoli. The lever arm was then aligned with the axis of rotation of the knee joint and the anatomical reference was set at 90°. Isokinetic MVC was then determined for the eccentric knee flexors (eccKF) and concentric knee extensors (conKE) of each participant's dominant lower limb. Prior to data collection three familiarisation repetitions were performed at a submaximal intensity. Participants then performed one set of three maximal contractions through a full range of movement at speeds equivalent to $180°\cdot s^{-1}$ and $60°\cdot s^{-1}$ for both eccKF and conKE. Passive knee flexion/extension separated each repetition and a seated rest period of 60 s interspersed each set. No performance feedback was provided during any of the exercise trials. Peak torque (N·m) was identified from each maximal repetition and the highest value was used to signify MVC.

Intraclass correlation coefficient (ICC) analysis has previously been conducted for measures of peak torque obtained at speeds equivalent to $180°\cdot s^{-1}$ and $60°\cdot s^{-1}$, for both eccKF and conKE, using the same IKD as was used in the current study (Eustace, Page and Greig, 2018). An ICC of 0.85 and 0.88 was reported for eccKF at $180°\cdot s^{-1}$

and 60°·s^{-1}, respectively, with similar values of 0.87 and 0.91 also reported for conKE at 180°·s^{-1} and 60°·s^{-1}, indicating almost perfect reliability (Landis and Koch, 1977).

3.2.6. Perceptions of Muscle Soreness

Perceptions of muscle soreness were measured using a 100 mm visual analogue scale whereby 0 mm indicated “no pain” and 100 mm indicated “unbearable pain” (Close et al., 2004). The use of a visual analogue scale has been reported as highly reliable for the measurement of acute pain, with an ICC of 0.97 (95% confidence intervals = 0.96 to 0.98) calculated from 432 paired measures of pain obtained 1 min apart in a sample of 96 participants (Bijur, Silver and Gallagher, 2001). It was also reported that 90% of these values were reproducible within 9 mm, suggesting that a change of $\geq$ 10 mm would likely be required to indicate a worthwhile change in pain when using this methodological tool (Bijur, Silver and Gallagher, 2001).

Participants rated perceived muscle soreness following the completion of two concentric-eccentric body weight squats. Squat technique was demonstrated and familiarised pre-trial. Participants were instructed to rate overall muscle soreness before providing individual ratings for localised muscle soreness of the gastrocnemius, quadriceps, hamstrings, gluteals and tibialis anterior muscle groups.

3.2.7. Blood Collection and Analysis

Blood samples (11 mL) were collected using a 21-gauge needle (BD Vacutainer, USA) from an antecubital vein while participants lay in a supine position. Samples

were collected into either a 6 mL pre-treated vacutainer tube containing ethylenediaminetetraacetic acid (EDTA), or a 5 mL pre-treated vacutainer tube containing a serum coagulation activator (BD Vacutainer, USA). Prior to centrifugation, samples collected for serum analysis were first left to coagulate for 30 min at room temperature. Samples were then centrifuged at 1000 *g* for 10 min at 4 °C (Biofuge Primo R, Sorvall, Thermo Scientific, UK), with the resulting supernatant aliquoted and stored at -80 °C for subsequent analysis.

Plasma (EDTA) samples were later used to determine protein carbonyl (PC) concentration ($nmol \cdot mL^{-1}$) using a commercially-available colorimetric assay kit (Cayman Chemical, USA). In accordance with the manufacturer guidelines, 200 μL samples were analysed in duplicate utilising a 2,4-dintrophenylhydrazine (DNPH) reaction, with the amount of protein-hydrozone produced quantified spectrophotometrically at an absorbance of 370 nm (FLUOstar Omega, BMG Labtech, Germany). Protein carbonyl concentration ($nmol \cdot mL^{-1}$) for each sample was then calculated by inserting the corrected absorbance (CA) into the following equation:

$$\textbf{Protein Carbonyl } (nmol \cdot mL^{-1}) = [(CA)/ (0.011\ \mu M^{-1})]\ (500\ \mu L/200\ \mu L)$$

Concentrations of C-reactive protein (CRP) ($mg \cdot L^{-1}$), creatine kinase (CK) ($U \cdot L^{-1}$) and lactate dehydrogenase (LDH) ($U \cdot L^{-1}$) were also determined in the serum using a clinical chemistry analyser (Pentra c400, Horiba, France). Samples of 20 μL were analysed in duplicate using colorimetry and turbidimetry, with analysis conducted in accordance to the manufacturer guidelines. The CVs of each measure are as follows: PC = 9.1%, CRP = 7.4%, CK = 7.1%, LDH = 0.5%.

3.2.8. Statistical Analysis

Data was first analysed for normality and homogeneity using the Shapiro-Wilk test and Levene's test, respectively. Outliers in the data were also assessed via visual inspection of Box Plots, with cases greater than ± 3 SD treated as an outlier in subsequent analysis. If an outlier was present (i.e. an outlier was detected in each parallel group for measures of serum CRP), statistical analysis was conducted on data both with and without said outliers to determine whether this impacted upon the outcomes reported (Weisberg, 2014). For the analysis of the primary outcome variables (isokinetic MVC, perceptions of muscle soreness, plasma PC and serum CK, LDH and CRP) an analysis of covariance (ANCOVA) was conducted with baseline scores used as the covariate to determine differences between groups [4 mg·day^{-1} vs. 12 mg·day^{-1} vs. placebo]. A one-way analysis of variance (ANOVA) was then used to determine temporal changes across all groups [pre-exercise vs. immediately post-exercise vs. 24 h post-exercise vs. 48 h post-exercise] for each primary outcome variable. A one-way ANOVA was also used to compare group differences [4 mg·day^{-1} vs. 12 mg·day^{-1} vs. placebo] in exercise intensity [VO_2 and running speed] during the 30 min downhill run, as well as baseline measures of age, VO_{2peak}, isokinetic MVC for eccKF and conKE, body mass and body fat to determine whether each parallel group was successfully matched. A two-way group [4 mg·day^{-1} vs. 12 mg·day^{-1} vs. placebo] x time [5, 10, 15, 20, 25, 30] ANOVA was then conducted to determine differences in RPE_O, RPE_L and HR obtained during the downhill run. If a significant main effect or interaction was observed, *post-hoc* analysis was performed with a Bonferroni adjustment. Effect sizes (partial eta squared ($P\eta^2$)) were calculated and

classified as trivial (< 0.01), small (0.01-0.059) moderate (0.06-0.137) or large (≥ 0.138) (Cohen, 1988). Confidence intervals (± 95%) were also calculated and are reported where necessary. Descriptive data are displayed as mean ± SD unless otherwise stated. Statistical analysis for all data was conducted using a statistical software package (SPSS, Version 25, USA), with significance accepted at $p < 0.05$.

3.3. Results

3.3.1. Isokinetic Maximal Voluntary Contraction Strength

At a movement speed of 180°·s^{-1} the decrease in eccKF MVC was not influenced by astaxanthin supplementation immediately post-exercise (4 mg·day^{-1}: 138.0 ± 13.9 N·m vs. 12 mg·day^{-1}: 133.5 ± 13.9 N·m vs. placebo: 135.7 ± 14.2 N·m; $p = 0.798$, $P\eta^2 = 0.021$), 24 h post-exercise (4 mg·day^{-1}: 134.6 ± 20.0 N·m vs. 12 mg·day^{-1}: 126.9 ± 20.3 N·m vs. placebo: 124.9 ± 19.9 N·m; $p = 0.582$, $P\eta^2 = 0.050$), or 48 h post-exercise (4 mg·day^{-1}: 131.7 ± 22.5 N·m vs. 12 mg·day^{-1}: 132.8 ± 22.4 N·m vs. placebo: 129.8 ± 22.9 N·m; $p = 0.966$, $P\eta^2 = 0.003$) (Figure 3.1a). This was similar for measures of eccKF MVC obtained at 60°·s^{-1}, with no effect of astaxanthin reported immediately post-exercise (4 mg·day^{-1}: 129.8 ± 12.3 N·m vs. 12 mg·day^{-1}: 121.5 ± 12.2 N·m vs. placebo: 132.9 ± 12.3 N·m; $p = 0.180$, $P\eta^2 = 0.151$), 24 h post-exercise (4 mg·day^{-1}: 131.1 ± 20.0 N·m vs. 12 mg·day^{-1}: 127.2 ± 19.9 N·m vs. placebo: 115.6 ± 20.1 N·m; $p = 0.286$, $P\eta^2 = 0.112$) or 48 h post-exercise (4 mg·day^{-1}: 128.9 ± 20.5 N·m vs. 12 mg·day^{-1}: 129.6 ± 20.4 N·m vs. placebo: 124.8 ± 20.6 N·m; $p = 0.884$, $P\eta^2 = 0.012$) (Figure 3.1b).

At a movement speed of 180°·s^{-1} the decrease in conKE MVC was also not influenced by astaxanthin supplementation immediately post-exercise (4 mg·day^{-1}: 162.8 ± 16.5 N·m vs. 12 mg·day^{-1}: 162.6 ± 16.1 N·m vs. placebo: 165.0 ± 16.4 N·m; p = 0.950, Pη^2 = 0.005), 24 h post-exercise (4 mg·day^{-1}: 167.1 ± 16.8 N·m vs. 12 mg·day^{-1}: 161.3 ± 16.5 N·m vs. placebo: 155.5 ± 16.7 N·m; p = 0.414, Pη^2 = 0.085), or 48 h post-exercise (4 mg·day^{-1}: 170.3 ± 19.9 N·m vs. 12 mg·day^{-1}: 156.9 ± 19.5 N·m vs. placebo: 157.7 ± 19.8 N·m; p = 0.345, Pη^2 = 0.101) (Figure 3.1c). This was similar for measures of conKE MVC obtained at 60°·s^{-1}, with no effect of astaxanthin reported immediately post-exercise (4 mg·day^{-1}: 210.0 ± 19.8 N·m vs. 12 mg·day^{-1}: 199.7 ± 19.8 N·m vs. placebo: 201.7 ± 19.6 N·m; p = 0.557, Pη^2 = 0.057), 24 h post-exercise (4 mg·day^{-1}: 213.5 ± 22.3 N·m vs. 12 mg·day^{-1}: 194.0 ± 22.3 N·m vs. placebo: 187.5 ± 22.1 N·m; p = 0.076, Pη^2 = 0.228) or 48 h post-exercise (4 mg·day^{-1}: 218.5 ± 32.6 N·m vs. 12 mg·day^{-1}: 202.8 ± 32.7 N·m vs. placebo: 196.7 ± 32.4 N·m; p = 0.401, Pη^2 = 0.087) (Figure 3.1d).

3.3.2. Perceptions of Muscle Soreness

There was no effect of astaxanthin supplementation reported for perceptions of overall muscle soreness immediately post-exercise (4 mg·day^{-1}: 45 ± 23 AU vs. 12 mg·day^{-1}: 45 ± 23 AU vs. placebo: 54 ± 23 AU; p = 0.701, Pη^2 = 0.033), 24 h post-exercise (4 mg·day^{-1}: 59 ± 24 AU vs. 12 mg·day^{-1}: 49 ± 24 AU vs. placebo: 58 ± 24 AU; p = 0.667, Pη^2 = 0.038) or 48 h post-exercise (4 mg·day^{-1}: 47 ± 24 AU vs. 12 mg·day^{-1}: 44 ± 24 AU vs. placebo: 57 ± 24 AU; p = 0.541, Pη^2 = 0.057) (Figure 3.2a). This was

similar for perceptions of localised muscle soreness, with astaxanthin supplementation not influencing measures obtained immediately post-exercise (4 mg·day^{-1}: 37 ± 17 AU vs. 12 mg·day^{-1}: 34 ± 17 AU vs. placebo: 35 ± 17 AU; p = 0.949, Pη^2 = 0.005), 24 h post-exercise (4 mg·day^{-1}: 42 ± 17 AU vs. 12 mg·day^{-1}: 34 ± 17 AU vs. placebo: 34 ± 17 AU; p = 0.525, Pη^2 = 0.059) or 48 h post-exercise (4 mg·day^{-1}: 39 ± 17 AU vs. 12 mg·day^{-1}: 27 ± 17 AU vs. placebo: 35 ± 17 AU; p = 0.362, Pη^2 = 0.092) (Figure 3.2b).

3.3.3. Biomarkers of Oxidative Stress and Inflammation

There was no effect of astaxanthin supplementation on the increase in plasma PC observed immediately post-exercise (4 mg·day^{-1}: 35.85 ± 7.33 nmol·mL^{-1} vs. 12 mg·day^{-1}: 33.14 ± 7.27 nmol·mL^{-1} vs. placebo: 42.29 ± 7.53 nmol·mL^{-1}; p = 0.077, Pη^2 = 0.226), 24 h post-exercise (4 mg·day^{-1}: 35.04 ± 7.52 nmol·mL^{-1} vs. 12 mg·day^{-1}: 34.20 ± 7.46 nmol·mL^{-1} vs. placebo: 36.71 ± 7.73 nmol·mL^{-1}; p = 0.817, Pη^2 = 0.020) or 48 h post-exercise (4 mg·day^{-1}: 32.28 ± 6.52 nmol·mL^{-1} vs. 12 mg·day^{-1}: 30.18 ± 6.47 nmol·mL^{-1} vs. placebo: 34.59 ± 6.70 nmol·mL^{-1}; p = 0.459, Pη^2 = 0.075) (Figure 3.3a).

Concentrations of serum CRP were also unaffected by astaxanthin supplementation, with or without the presence of outliers, with no differences between groups reported immediately post-exercise (with outliers: 4 mg·day^{-1}: 0.23 ± 0.05 mg·L^{-1} vs. 12 mg·day^{-1}: 0.24 ± 0.05 mg·L^{-1} vs. placebo: 0.24 ± 0.05 mg·L^{-1}; p = 0.851, Pη^2 = 0.015; without outliers: 4 mg·day^{-1}: 0.22 ± 0.05 mg·L^{-1} vs. 12 mg·day^{-1}: 0.22 ± 0.05 mg·L^{-1}

vs. placebo: 0.21 ± 0.05 mg·L^{-1}; p = 0.979, Pη^2 = 0.002), 24 h post-exercise (with outliers: 4 mg·day^{-1}: 0.95 ± 0.62 mg·L^{-1} vs. 12 mg·day^{-1}: 0.80 ± 0.62 mg·L^{-1} vs. placebo: 0.75 ± 0.62 mg·L^{-1}; p = 0.780, Pη^2 = 0.023; without outliers: 4 mg·day^{-1}: 0.68 ± 0.39 mg·L^{-1} vs. 12 mg·day^{-1}: 0.61 ± 0.40 mg·L^{-1} vs. placebo: 0.76 ± 0.40 mg·L^{-1}; p = 0.786, Pη^2 = 0.026) or 48 h post-exercise (with outliers: 4 mg·day^{-1}: 0.60 ± 0.64 mg·L^{-1} vs. 12 mg·day^{-1}: 0.72 ± 0.64 mg·L^{-1} vs. placebo: 0.49 ± 0.64 mg·L^{-1}; p = 0.780, Pη^2 = 0.023; without outliers: 4 mg·day^{-1}: 0.39 ± 0.25 mg·L^{-1} vs. 12 mg·day^{-1}: 0.41 ± 0.25 mg·L^{-1} vs. placebo: 0.51 ± 0.25 mg·L^{-1}; p = 0.612, Pη^2 = 0.053) (Figure 3.3b).

3.3.4. Biomarkers of Muscle Damage

Astaxanthin supplementation did not attenuate the increase in serum CK reported immediately post-exercise (4 mg·day^{-1}: 202 ± 58 U·L^{-1} vs. 12 mg·day^{-1}: 207 ± 60 U·L^{-1} vs. placebo: 221 ± 59 U·L^{-1}; p = 0.790, Pη^2 = 0.022), 24 h post-exercise (4 mg·day^{-1}: 720 ± 371 U·L^{-1} vs. 12 mg·day^{-1}: 643 ± 387 U·L^{-1} vs. placebo: 761 ± 378 U·L^{-1}; p = 0.834, Pη^2 = 0.017) or 48 h post-exercise (4 mg·day^{-1}: 425 ± 243 U·L^{-1} vs. 12 mg·day^{-1}: 329 ± 254 U·L^{-1} vs. placebo: 414 ± 248 U·L^{-1}; p = 0.660, Pη^2 = 0.041) (Figure 3.4a).

Similarly, astaxanthin supplementation did not attenuate the increase in serum LDH reported immediately post-exercise (4 mg·day^{-1}: 423 ± 33 U·L^{-1} vs. 12 mg·day^{-1}: 427 ± 34 U·L^{-1} vs. placebo: 412 ± 33 U·L^{-1}; p = 0.615, Pη^2 = 0.045), 24 h post-exercise (4 mg·day^{-1}: 414 ± 41 U·L^{-1} vs. 12 mg·day^{-1}: 398 ± 42 U·L^{-1} vs. placebo: 398 ± 41

$U \cdot L^{-1}$; p = 0.642, $P\eta^2$ = 0.041) or 48 h post-exercise (4 $mg \cdot day^{-1}$: 377 ± 42 $U \cdot L^{-1}$ vs. 12 $mg \cdot day^{-1}$: 387 ± 42 $U \cdot L^{-1}$ vs. placebo: 389 ± 42 $U \cdot L^{-1}$; p = 0.805, $P\eta^2$ = 0.020) (Figure 3.4b).

3.3.5. Temporal Responses

There was a temporal response reported across all groups for post-exercise changes in isokinetic MVC ($p < 0.003$, $P\eta^2 > 0.174$), perceptions of muscle soreness ($p < 0.001$, $P\eta^2 > 0.620$), plasma PC ($p < 0.001$, $P\eta^2 = 0.248$) and serum CRP ($p < 0.001$, $P\eta^2 = 0.382$), CK ($p < 0.001$, $P\eta^2 = 0.686$) and LDH ($p < 0.001$, $P\eta^2 = 0.547$). As such, the use of a downhill run to induce significant levels of muscle damage in this study was deemed a success. Each of these temporal responses are signified by (#) in Figures 3.1-3.4 below.

3.3.6. Heart Rate, RPE_O and RPE_L

A main effect of time was present for HR ($p < 0.001$, $P\eta = 0.786$), RPE_O ($p < 0.001$, $P\eta = 0.727$) and RPE_L ($p < 0.001$, $P\eta^2 = 0.667$) with each variable progressively increasing throughout the 30 min downhill run. No [group x time] interactions ($p > 0.926$, $P\eta < 0.040$) or main effects of group ($p > 0.606$, $P\eta^2 < 0.044$) were reported for each of these variables (Figure 3.5).

3.3.7. Group Matching

Experimental groups were deemed as being successfully matched, with no significant differences reported between groups for downhill running speed or intensity ($p \geq 0.486$, $P\eta \leq 0.063$) or any of the baseline characteristics provided in Table 3.1 ($p \geq 0.353$, $P\eta \leq 0.090$).

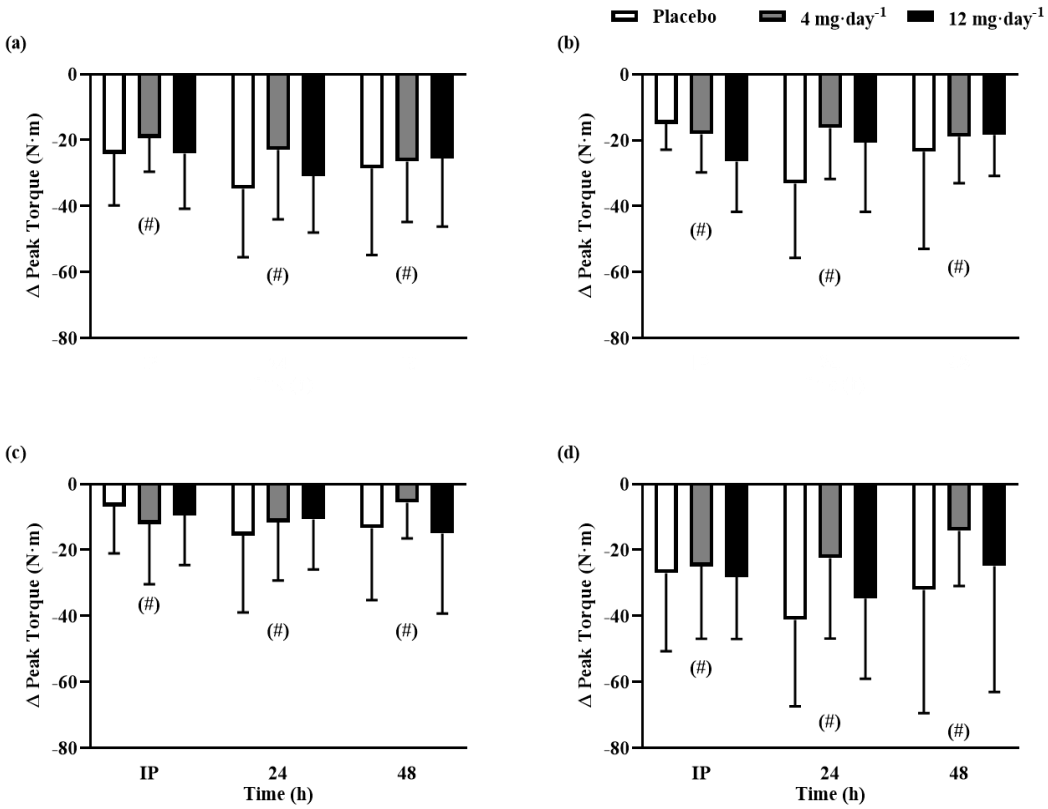

Figure 3.1 Unadjusted mean ± SD. Absolute differences in isokinetic maximal voluntary contraction strength (MVC) when compared to values obtained pre-exercise for the eccentric knee flexors (eccKF) at movement speeds of $180°\cdot s^{-1}$ (a) and $60°\cdot s^{-1}$ (b) and the concentric knee extensors (conKE) at movement speeds of $180°\cdot s^{-1}$ (c) and $60°\cdot s^{-1}$ (d). IP = immediately post-exercise, 24 = 24 h post-exercise and 48 = 48 h post-exercise, # denotes a significant difference to pre-exercise values ($p < 0.05$).

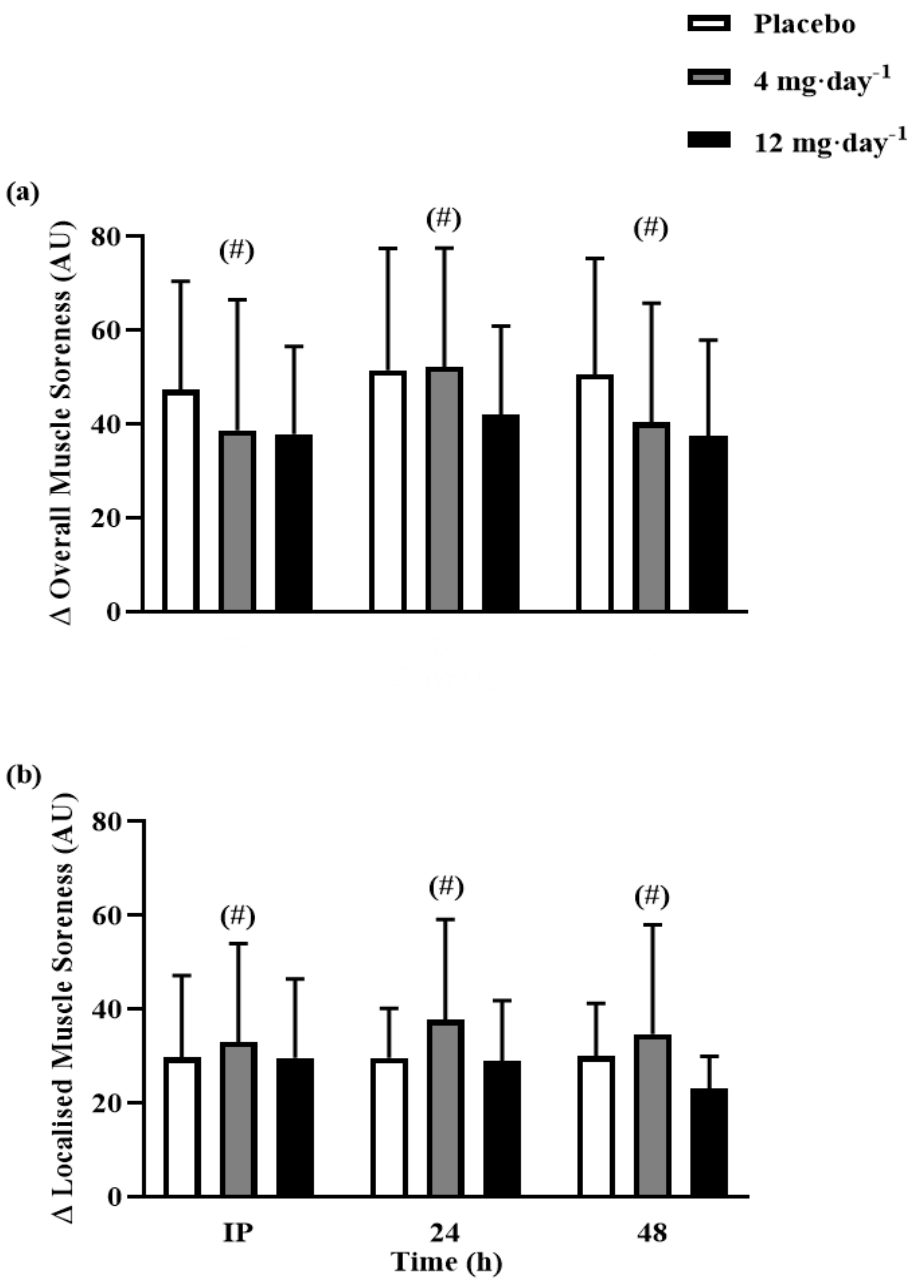

Figure 3.2 Unadjusted mean ± SD. Absolute differences in overall muscle soreness (a) and localised muscle soreness (b) when compared to values obtained pre-exercise. IP = immediately post-exercise, 24 = 24 h post-exercise and 48 = 48 h post-exercise, # denotes a significant difference to pre-exercise values ($p < 0.05$).

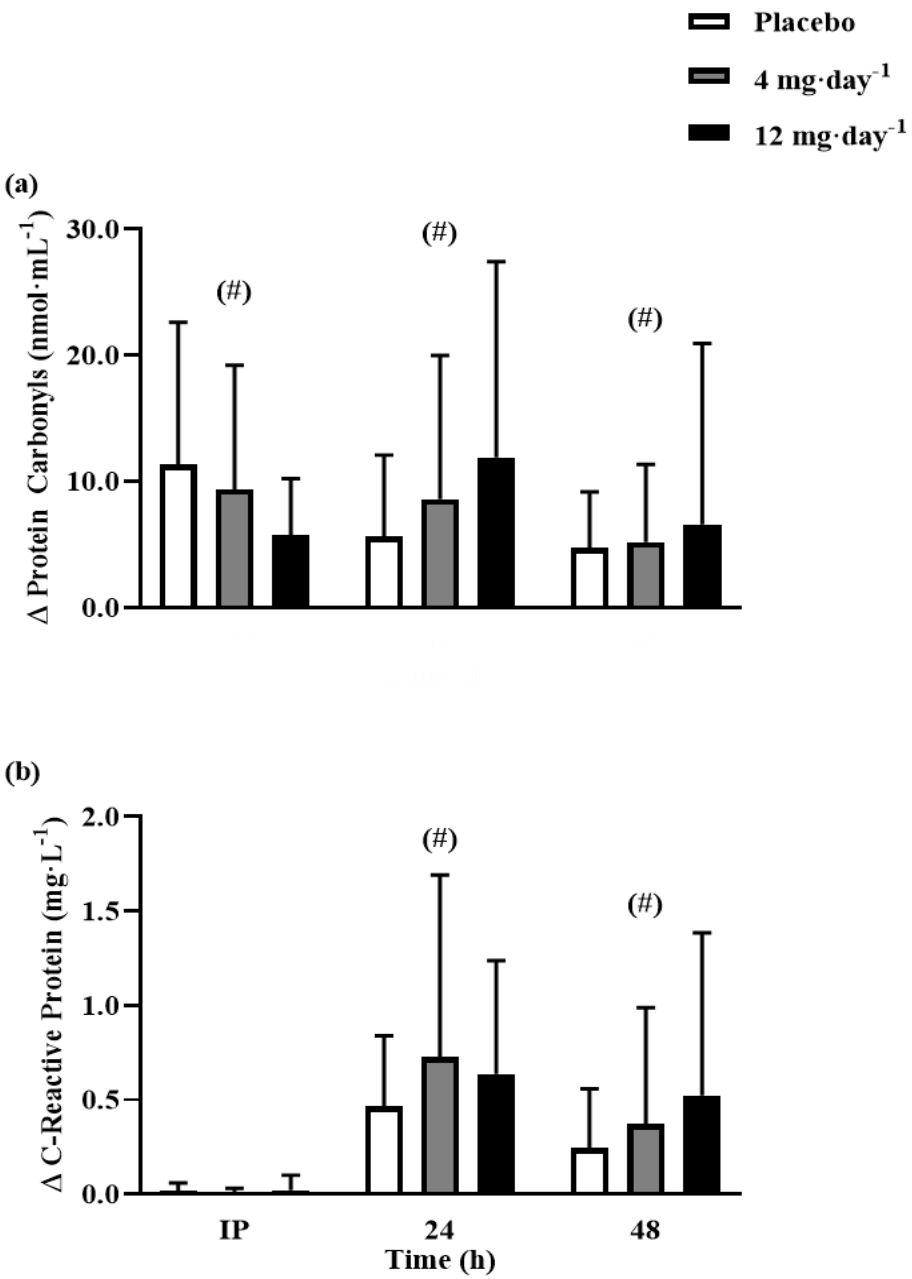

Figure 3.3 Unadjusted mean ± SD. Absolute differences in concentrations of protein carbonyls (PC) (a) and C-reactive protein (CRP) (b) when compared to values obtained pre-exercise. IP = immediately post-exercise, 24 = 24 h post-exercise and 48 = 48 h post-exercise, # denotes a significant difference to pre-exercise values ($p < 0.05$).

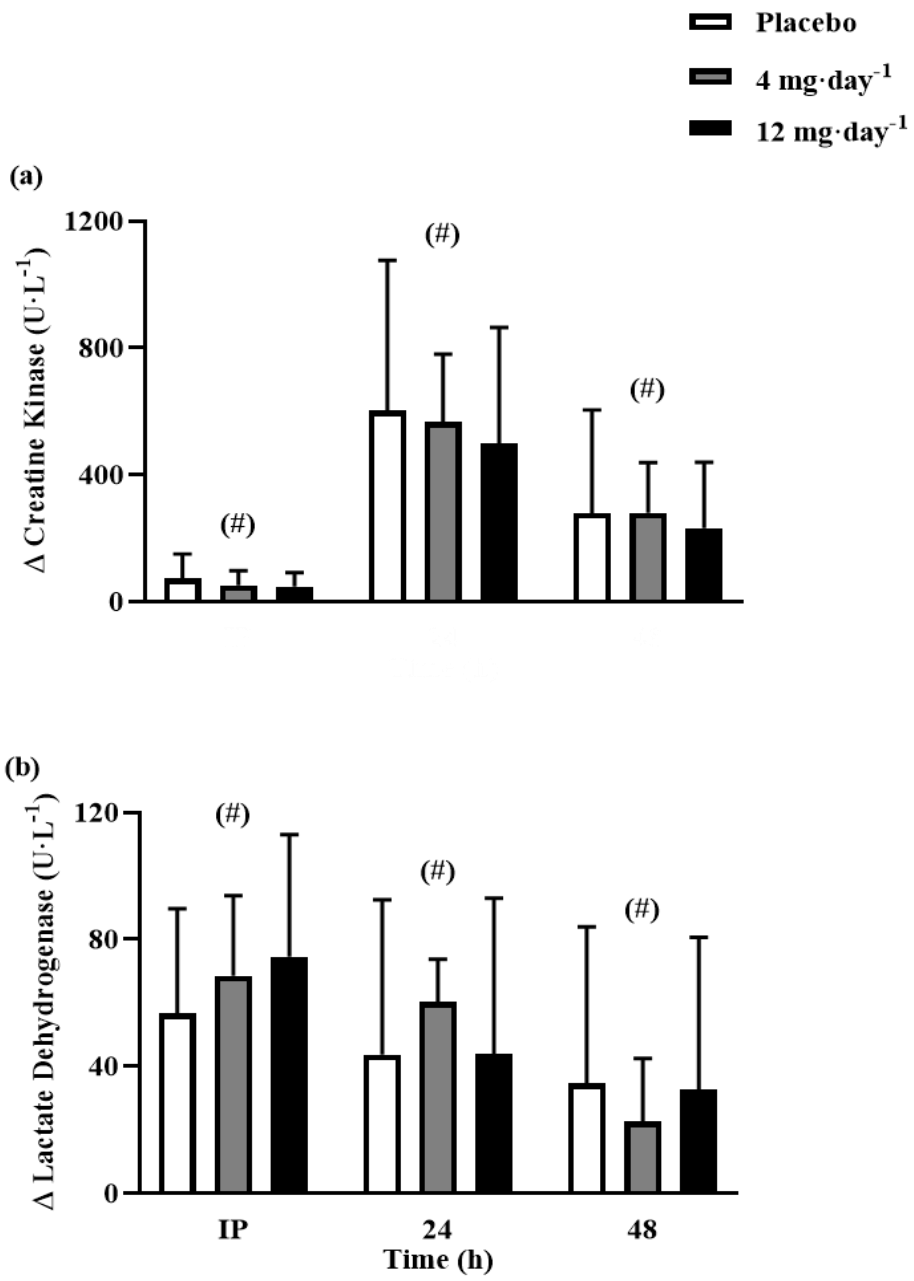

Figure 3.4 Unadjusted mean ± SD. Absolute differences in concentrations of creatine kinase (CK) (a) and lactate dehydrogenase (LDH) (b) when compared to values obtained pre-exercise. IP = immediately post-exercise, 24 = 24 h post-exercise and 48 = 48 h post-exercise, # denotes a significant difference to pre-exercise values ($p < 0.05$).

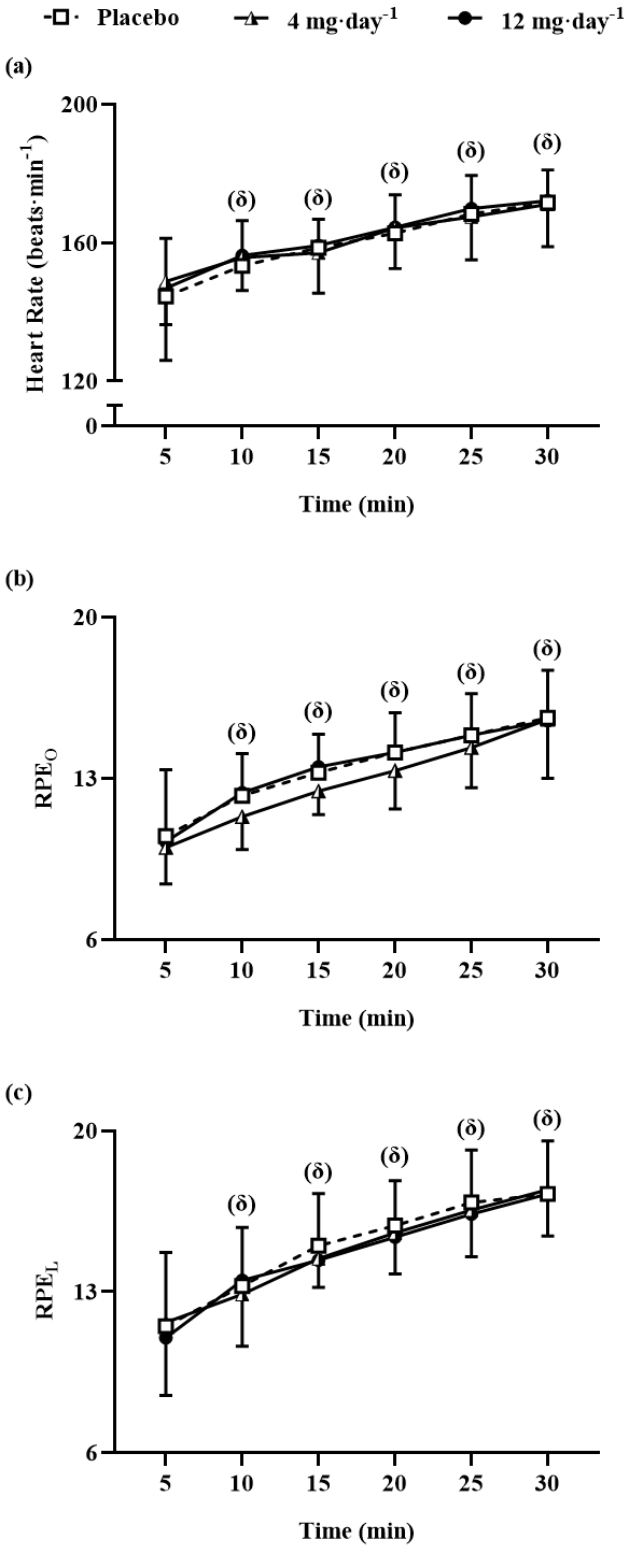

Figure 3.5 Mean ± SD. Heart rate (a) and ratings of perceived exertion for the whole-body (RPE_O) and the lower limbs (RPE_L) obtained over the duration of the 30 min downhill run. δ denotes a significant difference to the previous time point ($p < 0.05$).

Table 3.1 Mean ± SD. Participant characteristics used for group matching at baseline and for the speed and intensity the downhill run was completed at.

Variable	4 mg·day^{-1}	12 mg·day^{-1}	Placebo
Age (years)	25 ± 3	25 ± 4	28 ± 6
Body mass (kg)	80.5 ± 9.7	82.7 ± 9.6	81.5 ± 6.2
Body fat (%)	15.8 ± 5.3	16.8 ± 4.3	17.0 ± 2.2
VO_{2peak} (L·min^{-1})	4.06 ± 0.40	4.11 ± 0.52	3.97 ± 0.43
VO_{2peak} (mL·kg^{-1}·min^{-1})	50.9 ± 6.9	49.9 ± 5.5	48.8 ± 4.9
EccKF MVC at 180°·s^{-1} (N·m)	153.4 ± 29.7	154.5 ± 20.0	167.6 ± 25.2
EccKF MVC at 60°·s^{-1} (N·m)	144.0 ± 25.6	147.4 ± 19.6	153.0 ± 23.5
ConKE MVC at 180°·s^{-1} (N·m)	164.5 ± 31.6	172.9 ± 29.8	180.9 ± 25.7
ConKE MVC at 60°·s^{-1} (N·m)	220.1 ± 45.1	238.8 ± 44.0	228.0 ± 50.8
Downhill Running Speed (km·h^{-1})	13.6 ± 1.6	13.4 ± 2.6	13.0 ± 1.5
Downhill Running Intensity (% VO_{2peak})	69.3 ± 2.2	70.2 ± 1.2	70.4 ± 2.3

3.4. Discussion

The current investigation was conducted to explore the effect of astaxanthin supplementation on the muscle damage response to a 30 min downhill run in recreationally active males. In comparison to placebo, prior supplementation with 4 $mg \cdot day^{-1}$ or 12 $mg \cdot day^{-1}$ astaxanthin for 8 weeks did not attenuate the post-exercise increase in muscle damage and did not accelerate the recovery process in the 48 h post-exercise when compared to the placebo.

The findings of the current study are similar to those reported in resistance trained males following the completion of 10 sets of 10 eccentric knee extensions at 85% one repetition maximum (Bloomer et al., 2005). Resistance trained males were recruited to this study due to being a population that regularly trains with the volume and intensity required to induce muscle damage and potentially benefit from astaxanthin supplementation. Three weeks of 4 $mg \cdot day^{-1}$ astaxanthin supplementation, however, did not exert an effect on indices of muscle soreness, CK and the recovery of isokinetic and isometric MVC in the 92 h post-exercise when compared to the placebo (Bloomer et al., 2005). As resistance trained individuals were recruited, it was purported that the potential for astaxanthin to exert a recovery benefit may have been confounded by the repeated bout effect, whereby the protective adaptations induced by regular adherence to muscle damaging exercise would have reduced the muscle damage responses observed in this study (Proske and Allen, 2005). The current study, however, reports a similar outcome in recreationally active males unaccustomed to eccentric exercise and high-force resistance training, suggesting that training status does not confound the ability to detect changes in exercise-induced muscle damage. Indeed,

supplementation with 4 mg·day^{-1} and 12 mg·day^{-1} astaxanthin for 8 weeks did not exert an effect on similar measures of muscle soreness, CK and isokinetic MVC in the 48 h following a downhill run when compared to the placebo.

The findings of the current study are also similar to those reported in elite youth male soccer players following the completion of a 2 h bout of soccer exercise (n = 32; age: 17–18 years) (Djordjevic et al., 2012). A supplement effect of 4 mg·day^{-1} astaxanthin for 90 days, for example, was not reported in exercise-induced changes in plasma CK and generic measures of oxidative stress (TBARS, advanced oxidation protein products, superoxide anion, total antioxidant status, sulphydril groups, and SOD), suggesting a recovery effect was not present (Djordjevic et al., 2012). A similar study was later conducted in a separate cohort of elite youth male soccer players (n = 40; age: 17–18 years) (Baralic et al., 2015). In comparison to the exercising control, 4 mg·day^{-1} astaxanthin for 90 days was suggested to augment the training-induced reductions in both plasma CK (astaxanthin: −44.6%; control: −30.1%) and LDH (astaxanthin: −27.4%; control: −18.5%), while also attenuating training-induced increases in markers of inflammation, such as serum CRP and total leukocyte and neutrophil counts (Baralic et al., 2015). Caution is required however, as a significant supplement effect of astaxanthin was not reported in regard to each of the aforementioned measures, apart from the LDH biomarker ($p < 0.05$); suggesting that astaxanthin actually had little effect. Together these studies seemingly question the recovery potential of astaxanthin following the completion of muscle damaging exercise that has either a high-mechanical or a high-metabolic component.

When completing a bout of eccentric exercise, the initial muscle damage response is thought to be caused by the significant mechanical stress inflicted upon the exercising skeletal muscle and the concomitant increase in oxidative stress that results from this (Proske and Allen, 2005, Bowtell et al., 2011). This initial increase in oxidative stress and muscle injury is then thought to trigger a secondary inflammatory response which is thought to delay the recovery process and contribute to DOMS (Close et al., 2004, Mcleay et al., 2012). In the current study, measures of PC, CRP, CK and LDH were used as biomarkers of the oxidative stress, inflammatory and muscle damage responses to exercise. Indeed, increases in each of these biomarkers are commonly reported in response to exercise-induced muscle damage (Bloomer et al., 2007, Bowtell et al., 2011, Mcleay et al., 2012), a temporal response which was also observed in the current study (Figures 3.3 and 3.4). As astaxanthin did not provide a protective benefit to any of these biomarkers, with no differences reported between groups at any time point, the ability for astaxanthin to attenuate the initial and secondary phases of muscle damage are seemingly questioned. This outcome may also explain why astaxanthin did not attenuate the immediate and delayed changes in isokinetic MVC and perceptions of muscle soreness obtained at the same time points in the current study.

In sport nutrition and exercise physiology research, eccentric exercise protocols have received widespread use when aiming to quantify the recovery benefits of a particular intervention (Close et al., 2006, Bowtell et al., 2011, Mcleay et al., 2012, Hutchison et al., 2016). As the repeated bout effect is well-documented, however, parallel group designs are often relied upon, especially when whole-body exercise protocols are administered. This was the case in the current study, with participants matched based

upon baseline characteristics as described in 3.2.3. Group Matching. Despite no between group differences being observed statistically, with subtle differences also controlled using an ANCOVA, the response of bloodborne biomarkers and perceptions of muscle soreness to eccentric exercise are known to vary considerably between individuals (Bloomer et al., 2005, Bowtell et al., 2011).

To overcome this, single-limb muscle damaging exercise has been used as an alternative experimental design to mitigate the inter-individual variability often reported for exercise-induced muscle damage (Connolly et al., 2006, Bowtell et al., 2011). Experimental trials are counterbalanced for supplement and leg dominance to avoid a potential order effect, with the repeated bout effect reported as much smaller in the contralateral limb and to not confound the muscle damage responses observed in the 48 h following a second experimental trial (Howatson and van Someren, 2007, Bowtell et al., 2011). Future investigations into the recovery potential of astaxanthin should, therefore, consider utilising a single-limb experimental protocol so that a randomised crossover design can be employed. In doing so, each participant can act as their own control, minimising the confounding influence inter-individual variances in muscle damage could have upon outcome variables.

In conclusion, prior supplementation with 4 $mg \cdot day^{-1}$ or 12 $mg \cdot day^{-1}$ astaxanthin for 8 weeks did not provide a protection against exercise-induced muscle damage or accelerate the recovery process in recreationally active males following the completion of a 30 min downhill run. The uptake of astaxanthin was assumed, however, and not verified in the current investigation. Future research should seek to establish the pharmacokinetics of astaxanthin, while also implementing an

experimental protocol that enables the use of a randomised crossover design so that the potential recovery effect of astaxanthin can be explored in further detail.

4. The Effect of Two Doses of Astaxanthin Supplementation on the Fat Oxidative Capacity in Recreationally Active Males

4.1. Introduction

Increases in the fat oxidative capacity are often associated with numerous benefits pertaining to endurance performance and overall health (Fletcher et al., 2017). As such, methods capable of improving the fat oxidative capacity, both at rest and during exercise, continue to receive a plethora of research attention in sport, exercise and health nutrition (Hawley, Brouns and Jeukendrup, 1998, Yeo et al., 2011, Burke, 2015, Robinson et al., 2015, Fletcher et al., 2017). One potential method that has been investigated using a mice experimental model is astaxanthin supplementation. Indeed, evidence derived from mice would suggest that supplementation with astaxanthin for 3-5 weeks can increase the fat oxidative capacity during the completion of endurance exercise (Ikeuchi et al., 2006, Aoi et al., 2008). This is attributed to the potential for astaxanthin to protect the mitochondria from oxidative modifications induced by RONS during exercise, enabling an enhanced intercalation between FAT/CD36 and CPT1 on the mitochondrial membrane (Aoi et al., 2008).

In humans, however, a similar metabolic effect has yet to be replicated, with 4 weeks of astaxanthin supplementation (4 $mg \cdot day^{-1}$ and 20 $mg \cdot day^{-1}$) not influencing parameters of substrate metabolism (such as RER, carbohydrate and fat oxidation rates, and plasma glucose and NEFA concentrations) during 1-2 h of constant-load submaximal exercise in trained male individuals (Earnest et al., 2011, Res et al., 2013). It should be noted, that the rate at which fat is oxidised during exercise, and the exercise intensity at which this is maximal (FAT_{max}), is subject to large variation between individuals (Goedecke et al., 2000, Venables, Achten and Jeukendrup, 2005). In 300 healthy men and women, for example, FAT_{max} values in the range of 25 and

77% VO_{2max} were reported despite an average value of 48 ± 1% VO_{2max} reported overall (Venables, Achten and Jeukendrup, 2005). Consequently, the ability for previous research (Earnest et al., 2011, Res et al., 2013) to uncover a potential metabolic effect of astaxanthin during constant-load submaximal exercise may have been constrained by some individuals exercising above and others below the intensity at which MFO is elicited. For this reason, the current study utilises a FAT_{max} protocol so that potential changes in whole-body fat oxidation can be determined over a wide range of exercise intensities throughout supplementation (Achten, Gleeson and Jeukendrup, 2002).

Res et al. (2013) also detected no measurable changes in the plasma concentrations of the lipid peroxide malondialdehyde (MDA) during exercise, suggesting an antioxidant effect of astaxanthin was not present. Although speculative, this observation may provide a further insight as to why a metabolic effect was not reported in this study, with previous research suggesting an indirect metabolic modulation is mediated through the potent antioxidant function of astaxanthin within the mitochondrial membrane (Aoi et al., 2008). Concentrations of MDA are, however, reported to decrease significantly in individuals supplementing with 20 $mg \cdot day^{-1}$ astaxanthin for both 8 and 12 weeks, respectively (Choi, Youn and Shin, 2011). Likewise, 12 weeks of 8 $mg \cdot day^{-1}$ astaxanthin supplementation also attenuated markers of lipid peroxidation in healthy males (Karppi et al., 2007), potentially suggesting that a supplementation period of $\geq$ 8 weeks may be necessary to elicit the metabolic potential of astaxanthin in a human cohort. As such, the current study implements both a 4 week and an 8 week supplementation protocol with the aim of investigating whether long

term supplementation with astaxanthin (4 mg·day^{-1} and 12 mg·day^{-1}) can increase the fat oxidative capacity during the completion of a FAT_{max} protocol in recreationally active males.

4.2. Methods

4.2.1. Participants

Thirty recreationally active males (age: 25 ± 5 years, height 1.78 ± 0.07 m, body mass 81.0 ± 9.6 kg, body fat 16.4 ± 3.8%) provided written informed consent and participated in the study. Participants were not permitted to supplement with additional antioxidants/vitamins alongside those provided in the current study. A list of commonly consumed astaxanthin-rich foods to avoid was also provided to limit the additional dietary intake of astaxanthin during supplementation. Participants were also instructed to refrain from strenuous exercise and the consumption of alcohol and caffeine in the 24 h preceding each visit (Rosenberg et al., 1978, Westerterp-Plantenga et al., 2006), and to enter the laboratory following an overnight fast (~ 10 h), with the exception of ingesting water to ensure euhydration. Compliance with the above procedures was checked via 24 h dietary recall, with participants asked to replicate dietary intake prior to each trial. All participants visited the laboratory at a similar time of day (± 1 h) on seven separate occasions (one preliminary trial and six experimental trials). A double-blind parallel-groups design was employed.

4.2.2. Preliminary Trial

All participants were familiarised with all testing equipment and procedures to be used to minimise both learning and anxiety effects. This involved the performance of a FAT_{max} protocol identical to those performed during each experimental trial. A description of this protocol is provided in section 4.2.3. Experimental Trials.

4.2.3. Experimental Trials

On entering the laboratory measures of height and body mass were obtained, with body composition also determined using air displacement plethysmography. Participants then completed a FAT_{max} protocol to volitional exhaustion on a motorised treadmill. Adapted from previously described and validated protocols (Achten, Venables and Jeukendrup, 2003, Fletcher et al., 2017, Randell et al., 2017), exercise commenced at a speed of 3.5 $km \cdot h^{-1}$ and a gradient of 1.0%. Speed was then increased by 1.0 $km \cdot h^{-1}$ every 3 min until a RER of 1.00 was reached, indicating that fat oxidation had become negligible (FAT_{min}) (Jeukendrup and Wallis, 2005). To enable the determination of VO_{2peak}, speed then remained constant and the gradient was increased by 1.0% every min until a 3.0% gradient was achieved. The gradient was then kept constant and the speed was increased by 1.0 $km \cdot h^{-1}$ until volitional exhaustion. Breath-by-breath expired air was collected for VO_{2peak} determination and was defined as the highest 30 s average of VO_2 recorded during the FAT_{max} protocol. Heart rate and RPE (Borg, 1982) were also collected throughout exercise. Participants completed the FAT_{max} protocol on two separate occasions at baseline, 4 weeks (± 2 days) and 8 weeks (± 2 days) so that CV calculations could be made (CV = SD/mean),

with the average of the two measurements taken at each time point. Each trial was separated by $\geq$ 48 h.

During analysis, substrate utilisation rates were calculated using the VO_2 and VCO_2 data obtained during the final 60 s of each 3 min submaximal stage. The stoichiometric equations proposed by Jeukendrup and Wallis (2005) were used, assuming protein oxidation during exercise is negligible. Calculated fat oxidation rates were then plotted as a function of exercise intensity (% VO_{2peak} and % HR_{max}) and a 3rd degree polynomial curve with intersection at (0;0) was constructed for each participant (Stisen et al., 2006, Croci et al., 2014). Values for FAT_{max} (% VO_{2peak} and % HR_{max}), FAT_{min} (% VO_{2peak} and % HR_{max}), MFO ($g \cdot min^{-1}$) and MFO relative to fat free mass (MFO_{FFM}) ($mg \cdot kg\ FFM^{-1} \cdot min^{-1}$) were then determined, with the FAT_{zone} also calculated as the range of exercise intensities within 10% of MFO (Achten, Gleeson and Jeukendrup, 2002). To enable further comparisons, the lower and higher limits of the FAT_{zone} were calculated and are referred to as "FAT_{zone} low" and "FAT_{zone} high" (% VO_{2peak} and % HR_{max}) (Achten, Gleeson and Jeukendrup, 2002). The area under the curve (AUC) for total fat oxidised (g) was also calculated using the trapezoid method for both the FAT_{zone} (FAT_{zone} AUC) and the submaximal portion of the FAT_{max} protocol (submax AUC). The submaximal portion consisted of each 3 min stage of the FAT_{max} protocol completed prior to a RER of 1.00 being achieved.

4.2.4. Group Matching

Following the second baseline trial, participants were divided into three parallel groups, matched based upon the following characteristics; age (years), VO_{2peak}

absolute ($L \cdot min^{-1}$) and relative ($mL \cdot kg^{-1} \cdot min^{-1}$), FAT_{max} (% VO_{2peak} and % HR_{max}), MFO ($g \cdot min^{-1}$ and $mg \cdot kg\ FFM^{-1} \cdot min^{-1}$), body mass (kg) and body fat (%). Each group received one of three supplements, consisting of either 4 $mg \cdot day^{-1}$ astaxanthin (AstaReal®, Sweden), 12 $mg \cdot day^{-1}$ astaxanthin (AstaReal®, Sweden) or an appearance-matched placebo with no viable constituents (AstaReal®, Sweden). Participants ingested two capsules daily (one morning and one evening) for the entirety of the 8 week protocol, with compliance ensured via daily text message reminders and a pill count post-ingestion. To ensure the study remained double-blind, each supplement was provided with a randomised alphanumerical code until after data analysis was complete.

4.2.5. Statistical Analysis

Data was first analysed for normality using the Shapiro-Wilk test and standard graphical methods (Box Plots), and for homogeneity using the Levene's test. For the analysis of the primary outcome variables (FAT_{max}, MFO, FAT_{zone}, FAT_{min} and AUC) an ANCOVA was conducted with baseline scores used as the covariate to determine differences between groups [4 $mg \cdot day^{-1}$ vs. 12 $mg \cdot day^{-1}$ vs. placebo] after 4 weeks and 8 weeks of supplementation. To determine whether each parallel group was successfully matched, between group [4 $mg \cdot day^{-1}$ vs. 12 $mg \cdot day^{-1}$ vs. placebo] differences in baseline measures of age, VO_{2peak}, FAT_{max}, MFO, body mass and body fat were analysed using a one-way ANOVA. A two-way group [4 $mg \cdot day^{-1}$ vs. 12 $mg \cdot day^{-1}$ vs. placebo] x time [baseline and 8 weeks] ANOVA was also conducted to determine potential changes in training status (as inferred by VO_{2peak}, body mass and

body fat) during the 8 week supplementation period and whether inferred changes were different between groups. If a significant main effect or interaction was observed, *post-hoc* analysis was performed with a Bonferroni adjustment. Effect sizes ($P\eta^2$) were calculated and classified as trivial (< 0.01), small (0.01-0.059) moderate (0.06-0.137) or large (≥ 0.138) (Cohen, 1988). Confidence intervals (± 95%) were also calculated and are reported where necessary. Descriptive data are displayed as mean ± SD unless otherwise stated. Statistical analysis for all data was conducted using a statistical software package (SPSS, Version 25, USA), with significance accepted at $p < 0.05$.

4.3. Results

4.3.1. FAT_{max} and FAT_{min}

Astaxanthin supplementation did not exert an effect on the exercise intensity (% VO_{2peak} or % HR_{peak}) at which fat oxidation was reported to be maximal (FAT_{max}), with no significant differences reported between groups following 4 weeks (VO_2: $p = 0.973$, $P\eta^2 = 0.002$; HR: $p = 0.617$, $P\eta^2 = 0.037$) and 8 weeks (VO_2: $p = 0.939$, $P\eta^2 = 0.005$, HR: $p = 0.431$, $P\eta^2 = 0.063$) of supplementation (Table 4.1). Similarly, the exercise intensity (% VO_{2peak} or % HR_{peak}) at which fat oxidation became negligible (FAT_{min}) was not significantly different between groups following 4 weeks (VO_2: $p = 0.442$, $P\eta^2 = 0.061$; HR: $p = 0.718$, $P\eta^2 = 0.025$) and 8 weeks (VO_2: $p = 0.270$, $P\eta^2 = 0.096$, HR: $p = 0.465$, $P\eta^2 = 0.057$) of astaxanthin supplementation (Table 4.1).

4.3.2. Maximal Fat Oxidation Rates

There was no effect of astaxanthin supplementation on MFO ($g \cdot min^{-1}$), calculated as both a function of VO_2 and HR, with no significant differences reported between groups following 4 weeks (VO_2: $p = 0.826$, $P\eta^2 = 0.015$; HR: $p = 0.909$, $P\eta^2 = 0.007$) and 8 weeks (VO_2: $p = 0.987$, $P\eta^2 = 0.001$; HR: $p = 0.844$, $P\eta^2 = 0.013$) of supplementation (Table 4.2). There were also no significant differences reported between groups for measures of MFO_{FFM} ($mg \cdot kg\ FFM^{-1} \cdot min^{-1}$) obtained following 4 weeks (VO_2: $p = 0.870$, $P\eta^2 = 0.011$; HR: $p = 0.923$, $P\eta^2 = 0.006$) and 8 weeks (VO_2: $p = 0.956$, $P\eta^2 = 0.003$; HR: $p = 0.809$, $P\eta^2 = 0.016$) of astaxanthin supplementation (Table 4.2).

4.3.3. Area Under the Curve

There were no differences reported between groups for calculations of submax AUC (g) following 4 weeks (VO_2: $p = 0.679$, $P\eta^2 = 0.029$; HR: $p = 0.868$, $P\eta^2 = 0.011$) and 8 weeks (VO_2: $p = 0.962$, $P\eta^2 = 0.003$; HR: $p = 0.994$, $P\eta^2 < 0.001$) of astaxanthin supplementation (Table 4.3). Area under the curve calculations for total fat oxidised (g) within the FAT_{zone} (FAT_{zone} AUC) were also not significantly different between groups following 4 weeks (VO_2: $p = 0.732$, $P\eta^2 = 0.024$; HR: $p = 0.550$, $P\eta^2 = 0.045$) and 8 weeks (VO_2: $p = 0.987$, $P\eta^2 = 0.001$; HR: $p= 0.904$, $P\eta^2 = 0.008$) of astaxanthin supplementation (Table 4.3).

A similar outcome is reported for the lower and higher limits of the FAT_{zone} (% VO_{2peak} or % HR_{peak}). Indeed, values for FAT_{zone} low were not significantly different between

groups after 4 weeks (VO_2: p = 0.913, $P\eta^2$ = 0.007; HR: p = 0.784, $P\eta^2$ = 0.019) and 8 weeks (VO_2: p = 0.947, $P\eta^2$ = 0.004; HR: p = 0.682, $P\eta^2$ = 0.029) of astaxanthin supplementation, with values for FAT_{zone} high also unchanged between groups at the same time points (4 weeks: VO_2: p = 0.896, $P\eta^2$ = 0.008; HR: p = 0.537, $P\eta^2$ = 0.047; 8 weeks: VO_2: p = 0.884, $P\eta^2$ = 0.009; HR: p = 0.303, $P\eta^2$ = 0.088) (Table 4.3).

4.3.4. Baseline Group Matching

There were no significant differences reported between groups for any of the baseline characteristics provided in Table 4.4 ($p \geq 0.682$, $P\eta^2 \leq 0.028$). As such, baseline group matching was deemed successful.

4.3.5. Changes in Training Status

Participants were able to successfully maintain their training status over the 8 week supplementation period. This is inferred by there being no main effect of time ($p \geq 0.437$, $P\eta^2 \leq 0.023$), group ($p \geq 0.595$, $P\eta^2 \leq 0.038$) or a [time x group] interaction ($p \geq 0.436$, $P\eta^2 \leq 0.060$) for measures of VO_{2peak} ($L \cdot min^{-1}$ and $mL \cdot kg^{-1} \cdot min^{-1}$), body mass (kg) and body fat (%) obtained at baseline and 8 weeks, respectively (Table 4.5).

4.3.6. Coefficient of Variation Calculations

Calculations for the CV of each primary outcome variable are provided in Table 4.6 below.

Table 4.1 Mean ± SD. Adjusted values of FAT_{max} (% VO_{2peak} and % HR_{max}) and FAT_{min} (% VO_{2peak} and % HR_{max}) obtained following 4 weeks and 8 weeks of supplementation with either 4 mg·day^{-1} astaxanthin, 12 mg·day^{-1} astaxanthin or a placebo. "Difference" denotes the mean difference (± 95% CI) when compared to placebo. Baseline values for each variable were used as the covariate.

Variable	**Week 4**			**Week 8**		
	4 mg·day^{-1}	**12 mg·day^{-1}**	**Placebo**	**4 mg·day^{-1}**	**12 mg·day^{-1}**	**Placebo**
FAT_{max}						
VO_2	47.8 ± 4.3	47.5 ± 4.3	47.4 ± 4.3	47.7 ± 3.8	47.4 ± 3.8	47.1 ± 3.8
Difference	+0.4 (-5.4 to 5.0)	+0.1 (-4.9 to 5.0)		+0.3 (-4.1 to 4.7)	+0.6 (-3.8 to 4.9)	
HR	59.9 ± 4.7	57.9 ± 4.7	58.7 ± 4.6	60.6 ± 4.9	57.8 ± 4.9	59.7 ± 4.9
Difference	+1.1 (-4.1 to 6.5)	-0.9 (-6.2 to 4.5)		+0.9 (-4.7 to 6.5)	-1.9 (-7.5 to 3.7)	
FAT_{min}						
VO_2	86.9 ± 4.5	84.4 ± 4.6	86.4 ± 4.6	86.9 ± 5.4	83.7 ± 5.5	87.5 ± 5.5
Difference	+0.5 (-4.7 to 5.7)	-2.0 (-7.3 to 3.3)		+0.6 (-5.7 to 6.9)	-3.8 (-10.2 to 2.6)	
HR	92.4 ± 4.5	90.9 ± 4.5	91.2 ± 4.6	92.5 ± 4.7	90.9 ± 4.7	93.5 ± 4.7
Difference	+1.2 (-4.0 to 6.5)	-0.3 (-5.5 to 4.9)		-1.0 (-6.4 to 4.4)	-2.6 (-8.0 to 2.8)	

Table 4.2 Mean ± SD. Adjusted values of MFO ($g \cdot min^{-1}$) and MFO_{FFM} ($mg \cdot kg\ FFM^{-1} \cdot min^{-1}$) obtained following 4 weeks and 8 weeks of supplementation with either 4 $mg \cdot day^{-1}$ astaxanthin, 12 $mg \cdot day^{-1}$ astaxanthin or a placebo. "Difference" denotes the mean difference (± 95% CI) when compared to placebo. Baseline values for each variable were used as the covariate.

Variable	**Week 4**			**Week 8**		
	4 $mg \cdot day^{-1}$	**12 $mg \cdot day^{-1}$**	**Placebo**	**4 $mg \cdot day^{-1}$**	**12 $mg \cdot day^{-1}$**	**Placebo**
MFO						
VO_2	0.49 ± 0.07	0.48 ± 0.07	0.48 ± 0.07	0.46 ± 0.10	0.47 ± 0.10	0.46 ± 0.10
Difference	+0.02 (-0.07 to 0.10)	+0.00 (-0.08 to 0.09)		-0.01 (-0.12 to 0.11)	+0.00 (-0.11 to 0.12)	
HR	0.49 ± 0.07	0.47 ± 0.07	0.48 ± 0.07	0.45 ± 0.11	0.47 ± 0.11	0.46 ± 0.11
Difference	+0.01 (-0.08 to 0.09)	-0.01 (-0.09 to 0.07)		-0.02 (-0.14 to 0.11)	+0.01 (-0.12 to 0.13)	
MFO_{FFM}						
VO_2	7.23 ± 1.03	7.08 ± 1.03	7.00 ± 1.03	6.74 ± 1.45	6.92 ± 1.45	6.79 ± 1.45
Difference	+0.24 (-0.94 to 1.42)	+0.08 (-1.01 to 1.33)		-0.06 (-1.72 to 1.60)	+0.12 (-1.53 to 1.78)	
HR	7.14 ± 1.03	6.95 ± 1.03	7.06 ± 1.03	6.53 ± 1.53	6.97 ± 1.53	6.83 ± 1.53
Difference	+0.08 (-1.10 to 1.27)	-0.11 (-1.29 to 1.08)		-0.30 (-2.05 to 1.46)	+0.14 (-1.61 to 1.89)	

Table 4.3 Mean ± SD. Adjusted values of submax AUC (g), FAT_{zone} AUC (g), FAT_{zone} low (% VO_{2peak} or % HR_{max}) and FAT_{zone} high (% VO_{2peak} or % HR_{max}) obtained following 4 weeks and 8 weeks of supplementation with either 4 mg·day^{-1} astaxanthin, 12 mg·day^{-1} astaxanthin or a placebo. "Difference" denotes the mean difference (± 95% CI) when compared to placebo. Baseline values for each variable were used as the covariate.

Variable	**Week 4**			**Week 8**		
	4 mg·day^{-1}	**12 mg·day^{-1}**	**Placebo**	**4 mg·day^{-1}**	**12 mg·day^{-1}**	**Placebo**
Submax AUC						
VO_2	23.05 ± 4.52	21.76 ± 4.52	21.32 ± 4.52	21.40 ± 5.82	20.82 ± 5.82	20.73 ± 5.82
Difference	+1.73 (-3.45 to 6.90)	+0.44 (-4.74 to 5.61)		+0.67 (-6.00 to 7.34)	+0.10 (-6.57 to 6.76)	
HR	17.58 ± 3.60	16.89 ± 3.60	16.80 ± 3.60	17.11 ± 4.54	16.99 ± 4.53	16.88 ± 4.53
Difference	+0.79 (-3.34 to 4.91)	+0.09 (-4.03 to 4.22)		+0.23 (-4.96 to 5.42)	+0.11 (-5.08 to 5.29)	
FAT_{zone} AUC						
VO_2	11.98 ± 1.95	11.30 ± 1.95	11.53 ± 1.95	11.35 ± 2.60	11.19 ± 2.60	11.35 ± 2.60
Difference	+0.45 (-1.79 to 2.69)	-0.23 (-2.46 to 2.00)		-0.00 (-2.98 to 2.98)	-0.16 (-3.14 to 2.81)	
HR	10.51 ± 1.44	9.85 ± 1.43	9.94 ± 1.43	9.84 ± 2.19	9.91 ± 2.18	10.26 ± 2.17
Difference	+0.57 (-1.08 to 2.21)	-0.09 (-1.72 to 1.55)		-0.41 (-2.92 to 2.09)	-0.34 (-2.83 to 2.14)	

FAT_{zone} low						
VO_2	35.1 ± 4.2	35.3 ± 4.2	34.6 ± 4.2	34.7 ± 3.8	34.6 ± 3.8	34.2 ± 3.8
Difference	+0.5 (-4.3 to 5.4)	+0.8 (-4.0 to 5.6)		+0.5 (-3.8 to 4.9)	+0.5 (-3.9 to 4.8)	
HR	48.1 ± 5.0	46.5 ± 5.0	47.2 ± 5.0	48.3 ± 5.7	46.1 ± 5.7	47.6 ± 5.7
Difference	+0.9 (-4.9 to 6.7)	-0.7 (-6.4 to 5.0)		+0.7 (-5.8 to 7.2)	-1.5 (-8.0 to 5.0)	
FAT_{zone} high						
VO_2	60.0 ± 4.3	59.1 ± 4.3	59.8 ± 4.3	60.2 ± 3.8	59.3 ± 3.8	59.6 ± 3.7
Difference	+0.2 (-4.7 to 5.0)	-0.7 (-5.6 to 4.2)		+0.6 (-3.7 to 4.9)	-0.2 (-4.6 to 4.1)	
HR	70.8 ± 4.4	68.6 ± 4.4	69.6 ± 4.4	71.5 ± 4.3	68.6 ± 4.3	70.9 ± 4.3
Difference	+1.2 (-3.9 to 6.3)	-1.0 (-6.1 to 4.1)		-0.6 (-4.3 to 5.5)	-2.3 (-7.2 to 2.7)	

Table 4.4 Mean ± SD. Participant characteristics used for group matching at baseline.

Variable	4 mg·day^{-1}	12 mg·day^{-1}	Placebo
Age (years)	25 ± 3	25 ± 5	26 ± 7
Body mass (kg)	81.1 ± 10.1	79.2 ± 12.4	82.5 ± 6.1
Body fat (%)	16.4 ± 4.9	16.4 ± 4.1	16.4 ± 2.5
VO_{2peak} (L·min^{-1})	4.06 ± 0.43	4.03 ± 0.46	4.05 ± 0.47
VO_{2peak} (mL·kg^{-1}·min^{-1})	50.6 ± 6.5	51.5 ± 6.6	49.1 ± 4.8
FAT_{max} (% VO_{2peak})	46.0 ± 9.3	49.2 ± 9.3	47.3 ± 8.4
FAT_{max} (% HR_{max})	57.6 ± 11.0	61.0 ± 9.0	59.3 ± 10.3
MFO VO_2 (g·min^{-1})	0.49 ± 0.14	0.48 ± 0.13	0.49 ± 0.13
MFO HR (g·min^{-1})	0.50 ± 0.14	0.48 ± 0.13	0.49 ± 0.12
MFO_{FFM} VO_2 (mg·kg^{-1}·min^{-1})	7.28 ± 1.87	7.24 ± 1.54	7.07 ± 1.59
MFO_{FFM} HR (mg·kg^{-1}·min^{-1})	7.41 ± 1.92	7.21 ± 1.56	7.06 ± 1.42

Table 4.5 Mean ± SD. Groups characteristics used to infer potential changes in training status from pre- to post-supplementation.

Variable	4 mg·day^{-1}		12 mg·day^{-1}		Placebo	
	Baseline	**Week 8**	**Baseline**	**Week 8**	**Baseline**	**Week 8**
VO_{2peak} (L·min^{-1})	4.06 ± 0.43	4.08 ± 0.38	4.03 ± 0.46	4.04 ± 0.53	4.05 ± 0.47	4.01 ± 0.40
VO_{2peak} (mL·kg^{-1}·min^{-1})	50.6 ± 6.5	50.8 ± 6.5	51.5 ± 6.6	51.6 ± 6.5	49.1 ± 4.8	48.7 ± 4.3
Body mass (kg)	81.1 ± 10.1	81.0 + 9.2	79.2 ± 12.4	79.0 ± 11.5	82.5 ± 6.1	82.4 ± 6.0
Body fat (%)	16.4 ± 4.9	16.1 ± 5.1	16.4 ± 4.1	16.1 ± 4.4	16.4 ± 2.5	16.6 ± 2.1

Table 4.6 Overview of CV calculations for each primary outcome variable measured during the current study.

Variable	Mean	SD	CV
FAT_{max} (% VO_{2peak})	48.3	3.6	7.5%
FAT_{max} (% HR_{max})	60.1	3.3	5.5%
FAT_{min} (% VO_{2peak})	86.7	4.5	5.2%
FAT_{min} (% HR_{max})	92.0	3.0	3.2%
MFO VO_2 ($g \cdot min^{-1}$)	0.48	0.06	13.2%
MFO HR ($g \cdot min^{-1}$)	0.48	0.06	13.4%
MFO_{FFM} VO_2 ($mg \cdot kg^{-1} \cdot min^{-1}$)	7.04	0.93	13.2%
MFO_{FFM} HR ($mg \cdot kg^{-1} \cdot min^{-1}$)	7.02	0.94	13.4%
VO_2 AUC submax (g)	22.17	4.04	18.2%
HR AUC submax (g)	17.60	2.98	16.9%
VO_2 AUC FAT_{zone} (g)	11.77	1.78	15.1%
HR AUC FAT_{zone} (g)	10.55	1.58	15.0%
FAT_{zone} low (% VO_{2peak})	35.4	3.5	9.9%
FAT_{zone} low (% HR_{max})	48.4	3.8	7.8%
FAT_{zone} high (% VO_{2peak})	60.6	3.6	5.9%
FAT_{zone} high (% HR_{max})	70.8	2.9	4.1%

4.4. Discussion

The current study aimed to investigate whether supplementation with 4 mg·day^{-1} or 12 mg·day^{-1} astaxanthin for 4 or 8 weeks could induce changes in the fat oxidative capacity as measured during the completion of a FAT_{max} protocol in recreationally active males. The study outcomes suggest that astaxanthin did not exert an effect on the fat oxidative capacity, with indices relating to MFO and FAT_{max} remaining unchanged following astaxanthin supplementation when compared to the placebo.

The use of a FAT_{max} protocol in the current study enabled the measurement of whole-body fat oxidation rates over a wide range of exercise intensities. Therefore, when compared to previous research (Earnest et al., 2011, Res et al., 2013), the ability to detect astaxanthin-induced changes in the fat oxidative capacity would not have been confounded by the high inter-individual variability often reported for measures of fat oxidation during exercise (Venables, Achten and Jeukendrup, 2005). Despite this key methodological difference, the outcome of the current study is similar to those reported during the completion of a single-intensity, steady-state preload (Earnest et al., 2011, Res et al., 2013). Indeed, 4 weeks of 4 mg·day^{-1} astaxanthin supplementation has been previously reported to not influence measures of RER or whole-body fat and carbohydrate oxidation rates obtained during a 2 h submaximal cycle at 5% below the lactate threshold (Earnest et al., 2011). Likewise, 20 mg·day^{-1} astaxanthin for 4 weeks did not influence measures of RER or whole-body fat or carbohydrate oxidation rates obtained during the completion of a 1 h steady-state cycle at 50% W_{max} (Res et al., 2013). In the current study, similar outcomes are reported following doses of 4

$mg \cdot day^{-1}$ and 12 $mg \cdot day^{-1}$, with no differences in MFO, FAT_{max} or FAT_{zone} reported during a FAT_{max} protocol following both 4 weeks and 8 weeks of supplementation.

The metabolic findings of this study are, therefore, in contrast with those typically reported in mice exercise models following 3-5 weeks of astaxanthin intake (Ikeuchi et al., 2006, Aoi et al., 2008). Supplementation with 30 $mg \cdot kg^{-1}$ astaxanthin for 3 weeks, for example, was reported to significantly increase concentrations of plasma NEFA during a 15 min swim against an additional 5% body mass (Ikeuchi et al., 2006). In the same study, an increase in fat utilisation was also inferred, with muscle glycogen concentrations significantly greater post-exercise in mice supplementing with either 6 $mg \cdot kg^{-1}$ or 30 $mg \cdot kg^{-1}$ astaxanthin for 5 weeks (6 $mg \cdot kg^{-1}$ group: 4.0 $mg \cdot g^{-1}$ vs. 30 $mg \cdot kg^{-1}$ group: 4.2 $mg \cdot g^{-1}$ vs. control group: 3.4 $mg \cdot g^{-1}$). Whole-body fat oxidation rates ($mg \cdot kg^{-1} \cdot min^{-1}$) have also been reported as 21% greater during the completion of a 60 min run (25 $m \cdot min^{-1}$) in mice supplementing with 0.02% $w \cdot w^{-1}$ astaxanthin for 4 weeks when compared to an exercising control (Aoi et al., 2008).

A simple explanation for the disparity in study outcomes between human and mice research could be the difference in astaxanthin dose administered to each species. In mice exercise models, for example, astaxanthin doses of 6 $mg \cdot kg^{-1}$ and 30 $mg \cdot kg^{-1}$ have been administered over a 3-5 week supplementation period (Ikeuchi et al., 2006). If the same dose were to be administered in the current study, participants would have supplemented with either 486 $mg \cdot day^{-1}$ or 2430 $mg \cdot day^{-1}$ astaxanthin, doses that are far greater than the 4 $mg \cdot day^{-1}$ or 12 $mg \cdot day^{-1}$ that were safely administered. With Ikeuchi et al., (2006) also reporting a dose-response relationship between astaxanthin

supplementation and the fat oxidative capacity, this may partially explain why a metabolic effect of astaxanthin has yet to be replicated in exercising humans.

A limitation of the FAT_{max} protocol used in the current study is the variation reported, with primary outcome measures of MFO, FAT_{max}, FAT_{zone}, AUC and FAT_{min} having CVs in the range of 3.2-18.2% (4.3.6 Coefficient of Variation Calculations). Therefore, despite enabling the description of whole-body fat oxidation over a wide range of exercise intensities, the detection of small changes in fat oxidation may have been difficult due to the variation present. The use of a prolonged 8 week supplementation strategy also required the use of a parallel groups design, with participants matched based upon specific characteristics as described in 4.2.4. Group Matching. As such, participants were not able to act as their own control, with subtle differences in participant characteristics and individual responses to astaxanthin supplementation potentially confounding the ability to detect small changes in each outcome variable.

In conclusion, supplementation with 4 $mg \cdot day^{-1}$ or 12 $mg \cdot day^{-1}$ astaxanthin for 4 weeks and 8 weeks did not improve measures of exercising fat oxidative capacity in recreationally active males. This may have been implicated by the variation present in the experimental design/protocol or the differences in the astaxanthin doses administered between mice and human experimental models. Future research should aim to reduce the variation present in its outcome measures while also implementing a supplementation strategy that would allow participants to act as their own control, minimising the potential impact of confounding variables in the process.

5. Astaxanthin Supplementation Improves Performance and Fat Oxidation during a 40 km Cycling Time Trial

5.1. Introduction

Dietary supplementation strategies that can modify substrate utilisation patterns have received widespread attention in the literature, especially with regard to enhancing fat metabolism during exercise (Hawley, Brouns and Jeukendrup, 1998, Yeo et al., 2011, Burke, 2015). One such supplement is astaxanthin, a liposoluble carotenoid usually supplemented through the intake of *H. pluvialis*-derived antioxidant products. Based upon research conducted in mice, improvements in endurance performance are typically reported following 3-5 weeks of astaxanthin intake (Ikeuchi et al., 2006, Aoi et al., 2008, 2018). This is attributed to the potential for this liposoluble antioxidant to indirectly enhance the fat oxidative capacity during exercise through protecting the mitochondria from oxidative modifications induced by RONS (Aoi et al., 2008).

In humans, a similar ergogenic benefit was reported in trained cyclists, with 4 weeks of 4 mg·day^{-1} astaxanthin significantly improving 20 km cycling TT performance when compared to a placebo (mean improvement (MI) = astaxanthin: 121 s (5.1%) vs. placebo: 18 s (0.8%)) (Earnest et al., 2011). Conversely, in a 1 h cycling TT an ergogenic benefit was not reported following a 4 week supplementation with either 20 mg·day^{-1} astaxanthin (MI = 74 s (2.1%)) or an appearance-matched placebo (MI = 52 s (1.4%)) in trained cyclists or triathletes (Res et al., 2013). Although there is no clear explanation for the disparity between the two findings, it should be noted that astaxanthin did not influence measures of substrate utilisation obtained in either study (Earnest et al., 2011, Res et al., 2013). This outcome was also replicated in Chapter 4, whereby supplementation with 4 mg·day^{-1} or 12 mg·day^{-1} astaxanthin for both 4 weeks and 8 weeks, respectively, did not influence substrate utilisation rates obtained

during the completion of a FAT_{max} protocol in recreationally active, male participants. As the enhancement of the fat oxidative capacity is the mechanism by which astaxanthin is purported to exert its ergogenic potential, the absence of this effect may implicate an alternative ergogenic mechanism in Earnest et al. (2011), or partially explain the absence of a performance effect in Res et al. (2013).

A 3-5 week supplementation strategy is seemingly advocated in mice-models when seeking to elicit the ergogenic potential of astaxanthin during endurance exercise (Ikeuchi et al., 2006, Aoi et al., 2008, 2018). In human performance studies, although equivocal findings are reported, one key methodological consistency to mice research is the 3-5 week supplementation strategy implemented by both Earnest et al. (2011) and Res et al. (2013), respectively. Interestingly, plasma astaxanthin concentrations are reported to peak within the first week of intake, even when consumption is chronic. Rüfer et al. (2008), for example, quantified the uptake of ~ 1.25 $mg \cdot day^{-1}$ astaxanthin in the plasma of 28 healthy males over a 4 week period and reported a peak in concentration following 6 days of intake (Rüfer et al., 2008). This finding enables shorter supplementation periods to be advocated, which in turn may allow the use of a randomised crossover study design.

As such, the current study implements a shorter 7 day supplementation period, interspersed by 14 days of washout, to ensure that participants could act as their own control. A 40 km cycling TT was also used as a reliable measure of endurance performance obtained during a race distance that is common in competitive cycling events (Palmer et al., 1996, Laursen, Shing and Jenkins, 2003, Currell and Jeukendrup, 2008), increasing the ecological validity of the performance outcomes reported when

compared to studies conducted previously (Earnest et al., 2011, Res et al., 2013). Therefore, the aim of the current study was to investigate whether supplementation with 12 $mg \cdot day^{-1}$ astaxanthin for a shorter 7 day period can improve exercise performance and metabolism during the completion of a 40 km cycling TT in recreationally trained male cyclists using a randomised, crossover design.

5.2. Methods

5.2.1. Participants

Twelve recreationally trained male cyclists (age: 28 ± 6 years, height: 1.78 ± 0.07 m, body mass: 78.3 ± 7.6 kg, body fat: 13.7 ± 2.6%, VO_{2peak}: 56.5 ± 5.5 $mL \cdot kg^{-1} \cdot min^{-1}$, W_{max}: 347 ± 38 W) provided written informed consent and participated in the study. Participants were not permitted to supplement with additional antioxidants/vitamins alongside those provided in the current study. A list of commonly consumed astaxanthin-rich foods to avoid was also provided to limit the additional dietary intake of astaxanthin during supplementation. Participants were also instructed to refrain from strenuous exercise and the consumption of alcohol and caffeine in the 24 h preceding each visit (Rosenberg et al., 1978, Westerterp-Plantenga et al., 2006), and to enter the laboratory in a 4 h postprandial state, except for the ingestion of water to ensure euhydration. Compliance with the above procedures was checked via 24 h dietary recall, with participants asked to replicate dietary intake prior to each trial. All participants visited the laboratory on four separate occasions (two preliminary trials and two experimental trials) at a similar time of day (± 1 h). A randomised, double-blind, crossover design was employed.

5.2.2. Preliminary Trials

During the first preliminary visit participants completed a GXT to volitional exhaustion using an electromagnetically braked cycle ergometer. Following 5 min of unloaded pedalling, the GXT commenced at 75 W and work rate was increased by 30 W every 1 min until volitional exhaustion. Participants selected a preferred cadence to maintain throughout, with volitional exhaustion defined as an inability to maintain $\geq$ 60% of this cadence for $\geq$ 5 s despite strong verbal encouragement. Breath-by-breath expired air was collected for VO_{2peak} determination and was defined as the highest 30 s average of VO_2 recorded during the GXT. Heart rate and RPE (Borg, 1982) for the whole-body (RPE_O) and the lower limbs (RPE_L) were also collected throughout the test. A familiarisation with the 40 km TT and the experimental procedures employed was then undertaken during a second preliminary visit to ensure participants were accustomed to procedures employed during each experimental trial.

The importance of an initial familiarisation has been demonstrated in trained, male cyclists following the completion of a 40 km TT on three separate occasions (Laursen, Shing and Jenkins, 2003). Indeed, when performance data was compared across all three TTs a CV of 3.0 ± 2.9% was reported, however, when performance data was compared between the second and third TT only, a markedly lower CV of 0.9 ± 0.7% was reported. As such, the use of a familiarisation TT was strongly recommended to ensure the accurate and reliable assessment of 40 km TT performances, even when trained cyclists are recruited (Laursen, Shing and Jenkins, 2003).

Following the familiarisation trial participants received one of two randomised supplements to ingest for 7 days prior to the first experimental trial. A 14 day washout

period was then undertaken based upon estimations made using the pharmacokinetic data reported in Mercke Odeberg et al. (2003) and the calculations detailed in Saha (2018). Following this 14 day washout period, participants then supplemented with the second supplement for the 7 days preceding the second experimental trial. Further information detailing how these estimations were made are provided in section 2.4. (Treatment) above. Supplementation consisted of either 12 $mg \cdot day^{-1}$ astaxanthin (AstaReal®, Sweden) or an appearance-matched placebo with no viable constituents (AstaReal®, Sweden). Participants ingested two capsules daily (one morning and one evening), with compliance ensured via daily text message reminders and a pill count post-ingestion. To ensure the study remained double-blind, each supplement was provided with a randomised alphanumerical code until after data analysis was complete.

5.2.3. Experimental Trials

On the day of each experimental trial, participants entered the laboratory and undertook a 5 min warm-up before completing a best effort 40 km cycling TT on a Velotron Racermate™ cycle ergometer (Velotron, USA). Preferred frame geometry (i.e. handlebar and saddle position) was selected by the participant and was replicated for each experimental trial. Information regarding cadence, gear and distance covered was received, with no other information or external encouragement provided. Time to complete and mean power were recorded for both the total distance and for each 10 km quartile during the TT. Heart rate, RPE_O, RPE_L and ratings of fatigue (ROF) (Micklewright et al., 2017) were measured every 10 km. A finger prick capillary blood

sample was also taken at rest and every 10 km during the TT and was immediately analysed for indices of blood lactate (Lactate Pro 2, Japan) (CV = 2.7-5.1% (Bonaventura et al., 2015)), glucose (Hemocue, Sweden) (CV = 1.8% (Rajadhyaksha, Rodriguez and Nichols, 2007)) and triglycerides (Reflotron, USA) (CV = 0.8-9.9% (Statland, 1990)). Breath-by-breath expired air was obtained during the 10^{th}, 20^{th}, 30^{th} and 40^{th} km of the TT. Respiratory gas data were then used to calculate whole-body fat and carbohydrate oxidation rates (FATox and CHox, respectively) using the method of Jeukendrup and Wallis (2005).

5.2.4. Statistical Analysis

Data was first analysed for normality and homogeneity using the Shapiro-Wilk test and the Levene's test, respectively. A paired *t*-test was then used to compare group differences [astaxanthin vs. placebo] in performance time and mean power, and to determine whether a trial order effect [Trial 1 vs. Trial 2] was present for both performance time and mean power. A two-way condition [astaxanthin vs. placebo] x time [10, 20, 30, 40 km] ANOVA was used to determine differences in performance variables (time and power), respiratory variables (FATox and CHox, RER, VO_2), perceptual variables (RPE_O, RPE_L, ROF) and HR. Blood metabolites (lactate, glucose, triglycerides) were also analysed using a two-way condition [astaxanthin vs. placebo] x time [rest, 10, 20, 30, 40km] ANOVA. If a significant main effect or interaction was observed, *post-hoc* analysis was performed with a Bonferroni adjustment. Effect sizes were calculated using Hedge's *g* for paired comparisons and were interpreted as trivial (< 0.20), small (0.20-0.49), moderate (0.50-0.79) or large (≥ 0.80) (Cohen, 1988). The

Hedge's *g* correction was used to mitigate the positive bias of the Cohen's *d* effect size when $n < 20$ (Lakens, 2013). Confidence intervals (± 95%) were also calculated and are reported where necessary. Descriptive data are displayed as mean ± SD. Statistical analysis was conducted using a statistical software package (SPSS, Version 25, USA), with significance accepted at $p < 0.05$.

5.3. Results

5.3.1. Performance Variables

Time to complete the 40 km TT (Figure 5.1a) was significantly improved from 70.76 ± 3.93 min in the placebo condition to 69.90 ± 3.78 min in the astaxanthin condition, which equates to a 1.2 ± 1.7% improvement in performance (MI = 51 ± 71 s, 95% CI = 6-96 s, $p = 0.029$, $g = 0.21$). Mean power (Figure 5.1c) was also significantly improved from 213.8 ± 29.0 W in the placebo condition to 219.9 ± 28.7 W in the astaxanthin condition, which equates to an improvement of 2.8 ± 4.1% (MI = 6.1 ± 9.5 W, 95% CI = 0.1-12.1 W, $p = 0.047$, $g = 0.20$). Participants were also significantly faster (-21 ± 28 s, 95% CI = -3 to -39 s, $p = 0.026$, $g = 0.34$; Figure 5.1b) and produced significantly greater power (+9.5 ± 13.9 W, 95% CI = 0.6-18.3 W, $p = 0.038$, $g = 0.32$; Figure 5.1d) between 20-30 km in the astaxanthin condition. No performance differences were reported during any other 10 km quartile ($p \geq 0.083$). No trial order was present for performance time (Trial 1: 70.41 ± 4.01 min vs. Trial 2: 70.25 ± 3.75 min, $p = 0.993$, $g = 0.04$) or mean power (Trial 1: 216.6 ± 29.4 W vs. Trial 2: 217.2 ± 28.6 W, $p = 0.996$, $g = 0.02$).

5.3.2. Respiratory Variables

A [group x time] interaction was observed for FATox ($p = 0.037$), whereby FATox was significantly greater between 39-40 km following astaxanthin supplementation (Figure 5.2c), increasing from 0.13 ± 0.04 g·min^{-1} in the placebo condition to 0.22 ± 0.05 g·min^{-1} in the astaxanthin condition ($+0.09 \pm 0.13$ g·min^{-1}, 95% CI = 0.00-0.17 g·min^{-1}, $p = 0.044$, $g = 0.52$). A similar [group x time] interaction was also observed for RER ($p = 0.007$), whereby RER was also significantly lower between 39-40 km following astaxanthin supplementation (Figure 5.2a), decreasing from 0.99 ± 0.02 in the placebo condition to 0.96 ± 0.01 in the astaxanthin condition (-0.03 ± 0.04, 95% CI = -0.01 to -0.06, $p = 0.024$, $g = 0.60$). For measures of CHox a [group x time] interaction was also present ($p = 0.037$), with CHox significantly greater at 39-40 km in both conditions ($p < 0.045$). There was, however, no differences reported between conditions for CHox at any time point during the TT ($p \geq 0.118$; Figure 5.2e).

5.3.3. Blood Metabolites

Lactate (Figure 5.2b) was significantly increased above baseline throughout the TT ($p \leq 0.001$) and was also significantly greater at 40 km compared to 30 km ($p = 0.002$). Glucose (Figure 5.2f), in contrast, was significantly lower throughout the TT when compared to baseline ($p \leq 0.003$), and triglycerides (Figure 5.2d) were significantly increased above baseline at 30 km ($p = 0.027$) and 40 km ($p = 0.002$), as well as being significantly greater at 40 km than at any other time point ($p \leq 0.003$). There were no differences between conditions for each of these blood metabolites ($p \geq 0.346$).

5.3.4. Perceptual Variables and Heart Rate

Ratings of fatigue ($p < 0.001$), RPE_O ($p < 0.001$) and RPE_L ($p < 0.001$) all increased progressively over time with no effect of condition ($p \geq 0.131$). A main effect of time was also present for HR ($p < 0.001$) and VO_2 ($p < 0.001$) in both conditions ($p \geq 0.338$), with HR significantly greater at 40 km than at each previous time point and VO_2 significantly greater at 30 km than at 20 km and at 40 km when compared to each previous time point (Table 5.1).

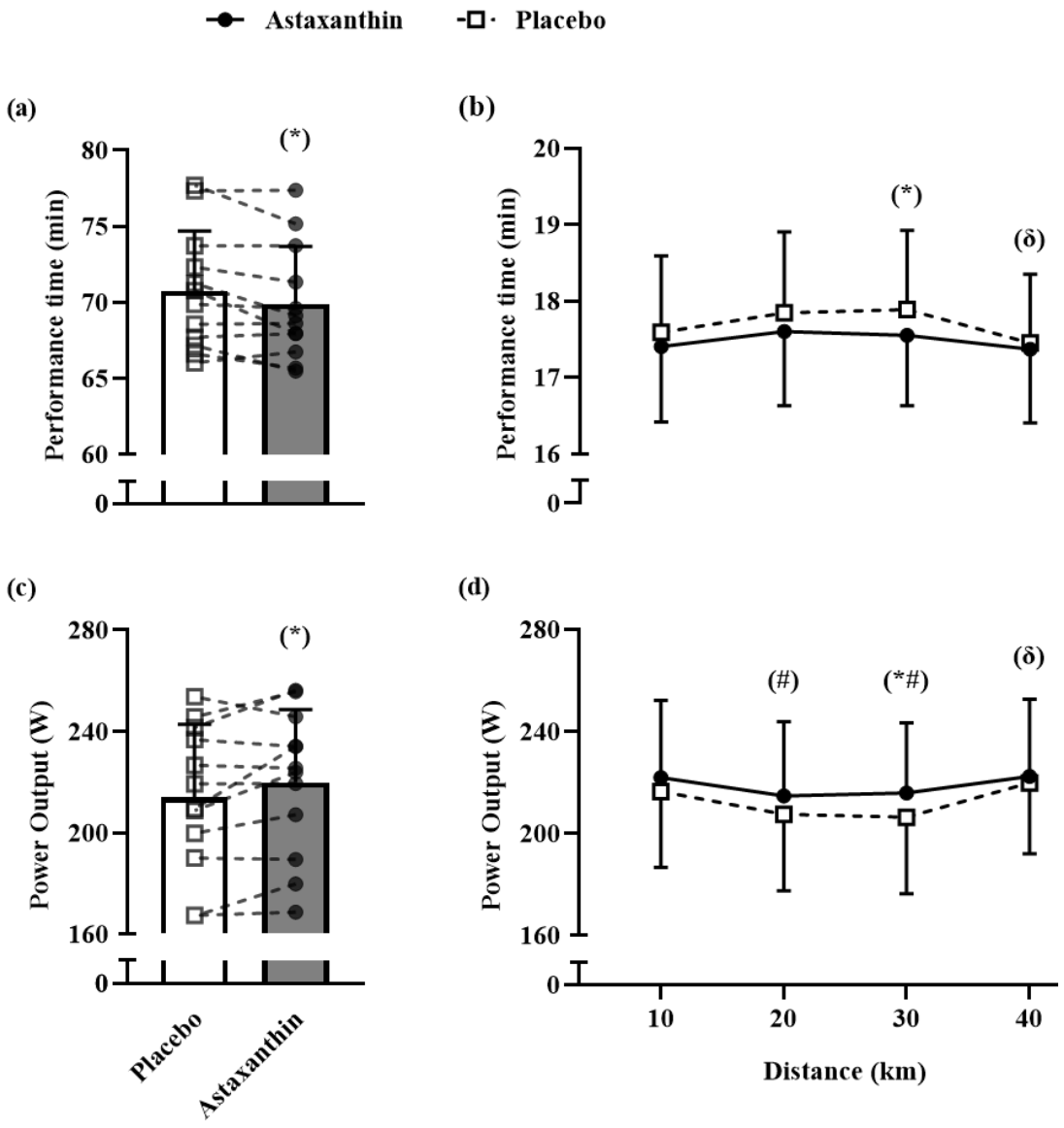

Figure 5.1 Mean ± SD. Individual values for performance time (a) and power output (c) during the 40 km time trial following each condition. Data for 10 km quartile performance times (b) and power outputs (d) are also displayed as mean (± SD) for each condition. * denotes a significant difference between conditions, # denotes a significant difference to the first time point, δ denotes a significant difference to the two previous time points ($p < 0.05$).

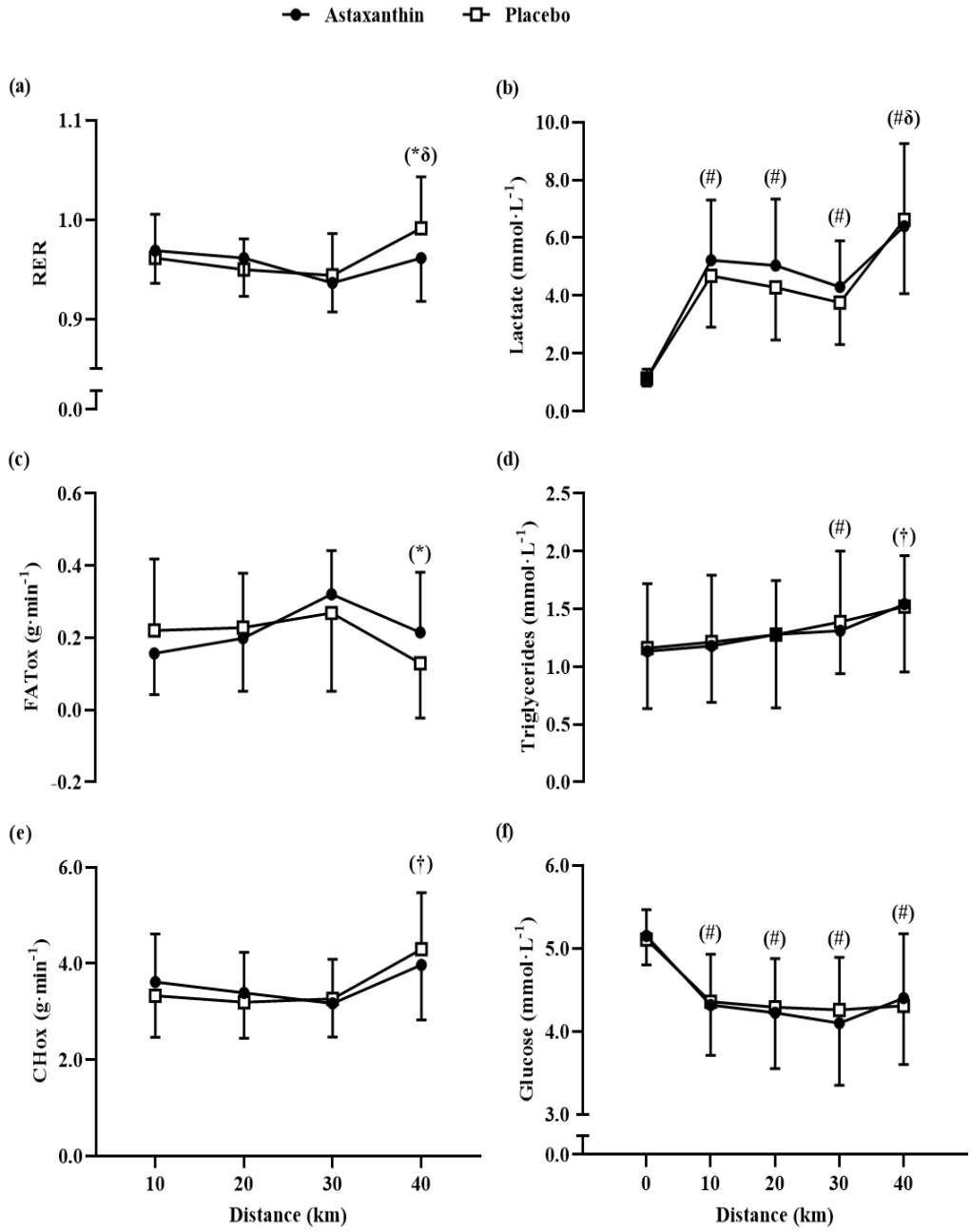

Figure 5.2 Mean ± SD. Respiratory measures of the respiratory exchange ratio (RER) (a), whole-body fat oxidation rates (FATox) (c), whole-body carbohydrate oxidation rates (CHox) (e), and blood metabolites lactate (b), triglycerides (d) and glucose (f) obtained over the duration of each 40 km time trial. * denotes a significant difference between conditions, # denotes a significant difference to baseline, δ denotes a significant difference to the previous time point, † denotes significant difference to all previous time points ($p < 0.05$).

Table 5.1 Mean ± SD. Physiological and perceptual results. δ denotes a significant difference to the previous time point, † denotes a significant difference to all previous time points ($p < 0.05$).

Variable	**Astaxanthin**				**Placebo**			
	10 km	**20 km**	**30 km**	**40 km**	**10 km**	**20 km**	**30 km**	**40 km**
VO_2 ($mL \cdot kg^{-1} \cdot min^{-1}$)	41.0 ± 7.6	40.1 ± 7.6	40.6 ± 8.2 $^{\delta}$	46.3 ± 8.6 †	39.6 ± 5.8	39.2 ± 6.9	41.1 ± 6.6 $^{\delta}$	45.2 ± 7.4 †
HR ($beats \cdot min^{-1}$)	153 ± 10	155 ± 9	156 ± 10	171 ± 10 †	153 ± 13	154 ± 11	156 ± 11	171 ± 9 †
ROF	3.7 ± 1.5	5.2 ± 1.3 $^{\delta}$	6.6 ± 1.3 $^{\delta}$	8.1 ± 1.6 †	3.2 ± 1.2	5.0 ± 1.5 $^{\delta}$	5.8 ± 1.7 $^{\delta}$	7.6 ± 1.8 †
RPE_O	13.8 ± 1.4	14.9 ± 1.4 $^{\delta}$	16.3 ± 1.4 $^{\delta}$	18.1 ± 1.3 †	13.3 ± 1.7	14.8 ± 1.5 $^{\delta}$	16.2 ± 1.5 $^{\delta}$	18.3 ± 1.8 †
RPE_L	14.9 ± 1.7	16.0 ± 1.2 $^{\delta}$	17.3 ± 1.2 $^{\delta}$	18.8 ± 0.8 †	14.8 ± 1.9	16.3 ± 1.5 $^{\delta}$	17.0 ± 1.3 $^{\delta}$	18.8 ± 0.8 †

5.4. Discussion

The current investigation is the first to demonstrate an increase in whole-body fat oxidation (FATox) and a corresponding reduction in RER during endurance exercise in humans supplementing with astaxanthin. This study also reports a small, yet significant, ergogenic benefit from 12 $mg \cdot day^{-1}$ astaxanthin supplementation for 7 days in recreationally trained male cyclists completing a 40 km cycling TT. This equates to a mean 51 s (1.2%) time improvement when compared to placebo.

The shorter 7 day supplementation strategy implemented in the current study enabled the use of a randomised crossover design and may provide a methodological insight as to why a metabolic effect of astaxanthin has been observed. In previous research, including Chapter 4, the application of a prolonged supplementation strategy has required the use of parallel group designs, with participants often matched based upon specific baseline demographics (i.e. fitness characteristics and anthropometry) (Earnest et al., 2011, Res et al., 2013). A potential advantage of the current study is, therefore, the ability to implement a randomised crossover design as this enabled each participant to act as their own control, minimising the potential impact subtle differences in participant characteristics and individual responses to astaxanthin could have upon the outcome variable (Cleophas and de Vogel, 1998, Burke and Peeling, 2018). As such, this would have improved the statistical power of the study and may have increased the ability to detect subtle differences in substrate utilisation during exercise, with FATox known to vary considerably between individuals of similar fitness demographics, even at the same absolute and relative exercise intensities (Achten, Gleeson and Jeukendrup, 2002, Venables, Achten and Jeukendrup, 2005).

The performance and metabolic findings of this study are consistent with those typically reported in mice following 3-5 weeks of astaxanthin intake (Ikeuchi et al., 2006, Aoi et al., 2008). Indeed, continuous improvements in swim TTE were reported from 1 week onwards in mice fed 6 or 30 $mg \cdot kg^{-1}$ astaxanthin for 5 weeks (Ikeuchi et al., 2006). Similarly, 0.02 $w \cdot w^{-1}$ astaxanthin for 4 weeks enhanced TTE at a running intensity of 30 $m \cdot min^{-1}$ in comparison to an exercising control (astaxanthin: 67.53 ± 4.20 min vs. control: 50.40 ± 5.00 min) (Aoi et al., 2008). In the same study, RER was also significantly lower from 20 min onward during a 60 min run (25 $m \cdot min^{-1}$), with FATox also higher in the astaxanthin group (Aoi et al., 2008). This highlights similarities with the present study following a smaller relative dose of astaxanthin (~ 0.15 $mg \cdot kg^{-1}$), with changes in FATox (+0.09 $g \cdot min^{-1}$) and RER (-0.03) only reported in the latter stages (39-40 km) of the 40 km cycling TT. Furthermore, with these changes occurring at the same relative exercise intensity, with no difference in VO_2 reported between groups at this time point (astaxanthin: 46.3 ± 8.6 $mL \cdot kg^{-1} \cdot min^{-1}$ vs. placebo: 45.2 ± 7.4 $mL \cdot kg^{-1} \cdot min^{-1}$), this provides further evidence to an increased utilisation of fat during this endurance performance event.

Possible explanations for these observations are received from previous exploratory research. Astaxanthin, for example, is known to accumulate in the mitochondrial membrane following consumption where it is suggested to indirectly enhance FATox during exercise through protecting CPT1 from RONS-induced oxidative modifications (Aoi et al., 2008, Wolf et al., 2010, Kidd, 2011). Alternatively, the expression of AMPK, is also reported to be upregulated following astaxanthin intake (Aoi et al., 2018). As a key enzyme in skeletal muscle metabolism, AMPK is

implicated in the stimulation of fatty acid oxidation; the transportation of fatty acids into the mitochondria, potentially through the intercalation of CPT1 and FAT/CD36; as well as the upregulation of many transcription factors, such as PGC-1α, that are known to promote mitochondrial biogenesis and control mitochondrial oxidative capacity (Thomson and Winder, 2009). As this mechanistic insight is received exclusively from *in vitro* and *in vivo* mice-models, it is clear that future exploratory research is necessary to elucidate similar mechanistic information in exercising humans, implementing performance tests of a similar or longer duration ($\geq$ 1 h) so that the ergogenic potential of astaxanthin can be explored in more detail.

The performance findings of this study are also consistent with those reported by Earnest et al. (2011), as 4 weeks of 4 mg·day^{-1} astaxanthin improved 20 km cycling TT performance in trained male cyclists (Earnest et al., 2011). Furthermore, the 121 s time improvement (5.1%) reported in the astaxanthin group was also significantly greater than the corresponding 18 s improvement (0.8%) reported in the placebo, suggesting a treatment effect was present (Earnest et al., 2011). In contrast, an ergogenic benefit was not reported during a 1 h cycling TT in trained male cyclists or triathletes following 4 weeks of supplementation with either 20 mg·day^{-1} astaxanthin (MI = 74 s (2.1%)) or an appearance-matched placebo (MI = 52 s (1.4%)) (Res et al., 2013). Although there is no clear explanation for the disparity between the two studies (Earnest et al., 2011, Res et al., 2013), it should be noted that neither Earnest et al. (2011) nor Res et al. (2013) reported differences in substrate utilisation during exercise, implicating an alternative ergogenic mechanism in Earnest et al. (2011), or partially explaining the absence of a performance effect in Res et al. (2013).

Four weeks of 4 mg·day^{-1} astaxanthin supplementation, for example, did not influence measures of RER, CHox or FATox obtained during a 2 h submaximal cycle at 5% below the lactate threshold (Earnest et al., 2011). Likewise, 20 mg·day^{-1} astaxanthin for 4 weeks did not influence measures of RER, CHox or FATox obtained during the completion of a 1 h steady-state cycle at 50% W_{max} (Res et al., 2013). As such, the increase in FATox and the decrease in RER reported in the latter stages of exercise in the current study are in contrast with previous research (Earnest et al., 2011, Res et al., 2013). A possible explanation for this is that the current study measured substrate utilisation during the completion of an ecologically valid performance event and not during a single-intensity, steady-state preload (Earnest et al., 2011, Res et al., 2013). As such, when compared to previous research (Earnest et al., 2011, Res et al., 2013), the metabolic measures obtained during the 40 km TT may have more accurately reflected the ergogenic mechanism by which astaxanthin is purported to improve performance during best effort endurance events.

Finally, as astaxanthin uptake was not quantified in the current investigation, the 7 day supplementation strategy was informed by previous literature that reported astaxanthin concentrations peak within the first week of intake, even when consumption is chronic (Rüfer et al., 2008). Nevertheless, an ergogenic and metabolic effect of astaxanthin was demonstrated following this shorter 7 day strategy, thus an exploration of the human pharmacokinetics of astaxanthin is clearly required so that an optimal supplementation strategy can be designed and implemented for future practice within this research area.

To conclude, supplementation with 12 mg·day^{-1} astaxanthin for 7 days provided an ergogenic benefit to 40 km cycling TT performance in recreationally trained male cyclists and enhanced whole-body fat oxidation in the final stages of this endurance-type performance event. Future research should seek to determine an optimal supplementation strategy for astaxanthin intake based on pharmacokinetics, while exploring the underlying mechanistic factors by which astaxanthin is purported to exert its ergogenic effect in exercising humans.

6. General Overview, Discussion and Conclusion

6.1. Overview

The aim of this thesis was to investigate the effects of astaxanthin supplementation on indices of exercise recovery, metabolism and performance in exercising humans. A summary of the outcomes reported in each experimental chapter are provided below:

Study 1 (Chapter 3): The aim of this study was to investigate whether prolonged supplementation with 4 mg·day^{-1} or 12 mg·day^{-1} astaxanthin for 8 weeks could attenuate various markers of muscle damage and improve the recovery of muscle function in the 48 h following a 30 min bout of eccentric exercise. This study recruited recreationally active males unaccustomed to eccentric exercise and high-force resistance training to ensure that the repeated-bout effect synonymous with eccentric exercise did not confound the study results. Despite this, the outcomes reported suggest that astaxanthin does not provide a recovery benefit following the completion of muscle damaging exercise, with no effect of supplement reported for any of the primary outcome measures obtained in this study.

Study 2 (Chapter 4): The aim of this study was to investigate whether 4 weeks or 8 weeks of astaxanthin supplementation (4 mg·day^{-1} and 12 mg·day^{-1}) could enhance the fat oxidative capacity, namely the MFO and FAT_{max}, in recreationally active males completing a FAT_{max} protocol. The use of a FAT_{max} protocol enabled this study to be the first to determine the effects of astaxanthin on whole-body fat oxidation during exercise of varying submaximal intensities. Similar outcomes were, however, reported to previous research, with astaxanthin not improving

measures of the fat oxidative capacity obtained during exercise after 4 weeks and 8 weeks of supplementation.

Study 3 (Chapter 5): The aim of this study was to investigate whether a shorter 7 day supplementation with 12 $mg \cdot day^{-1}$ astaxanthin could improve exercise performance and metabolism during a 40 km cycling TT in recreationally trained male cyclists. This study was the first conduct a randomised crossover design when investigating the ergogenic and metabolic effect of astaxanthin in exercising humans. This study was also the first to demonstrate an increase in whole-body fat oxidation and a simultaneous reduction in RER during endurance exercise in humans supplementing with astaxanthin. Furthermore, a small, yet significant, ergogenic benefit of 7 days astaxanthin supplementation was also reported, with a mean 51 s (1.2%) time improvement reported when compared to the placebo.

Together, the outcomes reported in this thesis demonstrate an ergogenic and a corresponding metabolic effect of astaxanthin supplementation; but also report a null effect of astaxanthin supplementation on indices of exercise-induced muscle damage and submaximal fat oxidation in recreationally active/trained male individuals.

A potential mechanistic explanation for these findings is received from previous exploratory research that has focussed on the function of astaxanthin in the mitochondrial membrane (Aoi et al., 2008, Wolf et al., 2010, Kidd, 2011). Astaxanthin, for example, is known to accumulate in the mitochondrial membrane following consumption, where it is reported to indirectly enhance whole-body fat oxidation during exercise through protecting CPT1 from RONS-induced oxidative modifications (Aoi et al., 2008, Wolf et al., 2010, Kidd, 2011). Astaxanthin is also

reported to upregulate the expression of AMPK (Aoi et al., 2018), a key enzyme implicated in the stimulation of fatty acid oxidation; the transportation of fatty acids into the mitochondria, potentially through the intercalation of CPT1 and FAT/CD36; as well as the upregulation of many transcription factors, such as PGC-1α, that are known to promote mitochondrial biogenesis and control mitochondrial oxidative capacity (Thomson and Winder, 2009).

As the mitochondria is an important predictor of endurance performance (Bishop et al., 2019), with its content and function significantly correlated with VO_{2max} (van der Zwaard et al., 2016) and TT performance (Granata et al., 2016), it is perhaps to be expected that a beneficial effect of astaxanthin supplementation would be reported in the experimental protocol (Chapter 5) that most accurately reflected this proposed mitochondrial mechanism. Future research should, therefore, be conducted to explore this ergogenic mechanism in more detail, implementing a performance TT of a similar or longer duration ($\geq$ 1 h) to the 40 km cycling TT implemented in Chapter 5.

The following sections are written to further discuss the general findings of this thesis, with limitations, practical implications and future directions for astaxanthin research also provided.

6.2. Differences Between Mice and Human Astaxanthin Research

It should first be noted that outcomes reported in an animal model are not always replicable in a human model (Shanks, Greek and Greek, 2009). Therefore, disparities in the outcomes reported between mice and human astaxanthin research may simply be explained by the genetic differences present between the two species.

Mice, for example, have a specific metabolic rate (metabolic rate per g of body mass) that is ~ 7 times greater than that of the average human (Demetrius, 2005). This relative increase in energy production is likely related to size-dependent differences in thermoregulation and heat loss, but also the increased requirement for nutrient supply (capillary density) and demand (mitochondrial density) in the muscle tissue of mice (Perlman, 2016). A consequence of this relative increase in metabolically active tissue and mitochondrial activity is the greater rate of RONS production and oxidative damage that typically occurs in mice when compared to humans (Demetrius, 2005, Perlman, 2016). With astaxanthin known to accumulate in the mitochondrial membrane following consumption and provide a protection against RONS-induced detriments to its function (Wolf et al., 2010, Kidd, 2011), the greater specific metabolic rate of mice may provide a genetic predisposition that partially explains why mice-models typically seem more responsive to astaxanthin supplementation than do humans.

An alternative explanation may be the difference in the astaxanthin dose administered in each of these experimental models. In mice, for example, astaxanthin doses of 6 $mg \cdot kg^{-1}$ and 30 $mg \cdot kg^{-1}$ have been administered over a 3-5 week supplementation period (Ikeuchi et al., 2006). If the same dose would have been administered in this thesis, participants (~ 80.4 kg body mass) would have been provided with either 482 $mg \cdot day^{-1}$ or 2412 $mg \cdot day^{-1}$ astaxanthin which are doses far greater than the 4 $mg \cdot day^{-1}$ (~ 0.05 $mg \cdot kg^{-1}$) or 12 $mg \cdot day^{-1}$ (~ 0.15 $mg \cdot kg^{-1}$) safely administered. With a dose-response relationship reported in mice for the beneficial effects of astaxanthin intake (Ikeuchi et al., 2006), it is perhaps unsurprising that beneficial effects of astaxanthin

are reported consistently in mice following higher relative doses, whereas similar effects remain equivocal in human research.

The ability for human research to increase the dose of astaxanthin administered is, however, potentially constrained by the safety recommendations currently advocated by EFSA (EFSA FEEDAP Panel, 2014, EFSA NDA Panel, 2014). Indeed, an ADI of 0.034 $mg \cdot kg^{-1} \cdot day^{-1}$ astaxanthin is currently advocated based upon research conducted in rats (EFSA FEEDAP Panel, 2014), which would equate to a dose of ~ 2.97 $mg \cdot day^{-1}$ in the current thesis. It was also concluded that the safety of 0.06 $mg \cdot kg^{-1} \cdot day^{-1}$ astaxanthin (~ 4.82 $mg \cdot day^{-1}$ in the current thesis) had yet to be fully established for human consumption (EFSA NDA Panel, 2014).

There were, however, no adverse effects reported by the participants recruited to this thesis in response to supplementation with 4 $mg \cdot day^{-1}$ or 12 $mg \cdot day^{-1}$ astaxanthin for 7 days, 4 weeks or 8 weeks, respectively. Furthermore, no adverse effects were reported previously following the acute intake of 40 mg astaxanthin (Mercke Odeberg et al., 2003), or the chronic supplementation of 6-40 $mg \cdot day^{-1}$ astaxanthin for 4-8 weeks (Spiller and Dewell, 2003, Kupcinskas et al., 2008, Res et al., 2013). The possible adverse effects measured in each of these studies ranged from headaches and perceptions of abdominal/stomach pain, to blood pressure and biochemical parameters, including a metabolic panel and cell blood count (Mercke Odeberg et al., 2003, Spiller and Dewell, 2003, Kupcinskas et al., 2008). Together, each of these studies suggest that it may be possible to safely advocate both acute and chronic intakes of astaxanthin that are considerably greater than the current ADI. Future research is required to further elucidate the safety of astaxanthin so that human

consumption guidelines can be adjusted accordingly, potentially enabling the use of an increased dose when seeking to elucidate the beneficial properties of astaxanthin in an exercising human cohort.

In mice experimental models, indices of substrate utilisation, oxidative stress and inflammation have also been measured in the skeletal and cardiac muscle in addition to the plasma and/or serum (Aoi et al., 2003, 2008, Lee et al., 2003, Ikeuchi et al., 2006, Liu et al., 2014). In comparison, human research (including the current thesis) has relied upon biomarkers obtained from the plasma/serum to provide a global snapshot of the effect of astaxanthin in exercising humans (Bloomer et al., 2005, Earnest et al., 2011, Djordjevic et al., 2012, Res et al., 2013, Baralic et al., 2015). This is a potential limiting factor to human research conducted thus far, with the extraction of muscle tissue in mice providing a more sophisticated means of detecting astaxanthin-induced changes in markers of substrate utilisation, oxidative stress and/or inflammation. Future research should, therefore, seek to obtain muscle biopsy data from exercising humans so that the effects of astaxanthin supplementation can be explored in more detail at an intramuscular level.

6.3. Experimental Study Designs

The current thesis has employed two experimental study designs to investigate the efficacy of astaxanthin supplementation in exercising humans. In Chapter 3 and Chapter 4 a parallel groups design was employed due to the use of a prolonged 8 week supplementation strategy, with participants matched based upon specific baseline characteristics as described in 3.2.3. Group Matching and 4.2.4. Group Matching. In

Chapter 5, the shorter 7 day supplementation strategy implemented enabled the use of a randomised crossover design, with participants acting as their own control.

Interestingly, a beneficial effect of astaxanthin was only reported in Chapter 5 of this thesis, with 7 days of 12 mg·day^{-1} astaxanthin supplementation significantly improving 40 km cycling TT performance and whole-body fat oxidation in recreationally trained male cyclists. This is contrast to Chapter 3 and Chapter 4, whereby supplementation with 4 mg·day^{-1} or 12 mg·day^{-1} astaxanthin for 4-8 weeks did not attenuate the muscle damage response to exercise or enhance the fat oxidative capacity during submaximal exercise in a similar demographic of recreationally active males.

With dose and participant demographics similar across studies, the use of a randomised crossover design in Chapter 5 may provide a methodological insight as to why this study was the only one to report a beneficial effect of astaxanthin, even though a shorter supplementation period was undertaken. In previous research, for example, the markers of muscle damage and substrate utilisation obtained were reported to vary considerably, even between individuals of a similar fitness demographic (Goedecke et al., 2000, Bloomer et al., 2005, Venables, Achten and Jeukendrup, 2005, Bowtell et al., 2011). A potential advantage of Chapter 5 was, therefore, the ability for each participant to act as their own control, minimising the potential impact subtle differences in participant characteristics and individual responses to astaxanthin could have upon the outcome variable (Cleophas and de Vogel, 1998, Burke and Peeling, 2018). As such, this may have increased the ability of Chapter 5 to detect subtle differences in substrate utilisation and performance

during exercise. Future research conducted in astaxanthin should, therefore, consider implementing a randomised crossover study design when aiming to detect subtle changes in exercise recovery, metabolism or performance as a result of astaxanthin supplementation.

6.4. Pharmacokinetics of Astaxanthin Uptake

Pharmacokinetic data is available from research that has quantified the uptake and elimination kinetics of acute astaxanthin supplementation in human plasma using high-performance liquid chromatography (Østerlie, Bjerkeng and Liaaen-Jensen, 2000, Mercke Odeberg et al., 2003, Coral-Hinostroza et al., 2004, Rüfer et al., 2008). The uptake kinetics of chronic supplementation, however, has only been analysed in humans by Rüfer et al. (2008) during the investigation of 28 healthy males over a 4 week period. Prior to study onset, astaxanthin concentrations were quantified as non-detectable, following which two randomised groups (n = 14 each group) consumed either 250 g of wild or aquacultured salmon daily, to obtain ~ 1.25 $mg \cdot day^{-1}$ astaxanthin (5 µg astaxanthin$\cdot g^{-1}$ salmon flesh). Following 6 days of consumption, astaxanthin concentrations reached a plateau of 33.7 ± 16.2 $nmol \cdot L^{-1}$ (wild salmon) and 52.4 ± 16.2 $nmol \cdot L^{-1}$ (aquacultured salmon), respectively, with concentrations reported to not significantly change for the remainder of the 4 week protocol (Rüfer et al., 2008).

It therefore appears that when the intake of astaxanthin is chronic, maximal concentrations can be achieved and maintained within the first week of intake, even when astaxanthin is obtained from different sources. This data, however, is collated

from one study only, where astaxanthin was consumed as part of the diet, requiring a daily intake of 250 g salmon (Rüfer et al., 2008). Future research should, therefore, aim to clarify the bioavailability of astaxanthin from a variety of different sources, including supplementation, with information regarding the elimination kinetics also of importance to understand how the availability of astaxanthin may diminish in humans over time. In doing so, an optimal supplementation strategy for astaxanthin intake in exercise humans can be devised, increasing the potential ability for the successful integration of astaxanthin into sport nutrition practices in the future.

A limitation of each study in this thesis is that the uptake of astaxanthin was not verified from pre- to post-supplementation. As such, the supplementation strategies implemented in this thesis were informed by previous astaxanthin literature (Rüfer et al., 2008, Earnest et al., 2011, Res et al., 2013). An interesting finding reported in this thesis, however, is that a beneficial effect of astaxanthin was only reported following a shorter 7 day supplementation, and not following prolonged intakes of 4 weeks or 8 weeks, respectively. As discussed in section 6.3 (Experimental Study Designs), this may simply be explained by 7 days of intake enabling the ability to conduct a randomised crossover design, minimising the potential impact subtle differences in participant characteristics and individual responses to astaxanthin could have upon the outcome variable. Without the uptake of astaxanthin being quantified, however, it is difficult to make a robust conclusion regarding the effect of astaxanthin on the outcome variables measured in this thesis. For example, it is uncertain as to whether the ergogenic effect reported in Chapter 5 was because astaxanthin was adequately taken up into the muscle. Similarly, it is uncertain as to whether the no effects reported in Chapter 3 and Chapter 4 were implicated by astaxanthin not being adequately taken

up into the exercising muscle. Although compliance was ensured via daily text message reminders and a pill count post-ingestion, future study should seek to confirm this uptake in the plasma/serum or the muscle of its participants.

A further limitation of each study in this thesis is that chronic dietary intake was not controlled or quantified beyond the 24 h period that preceded each experimental trial. Previously, the influence of 4-day dietary intake has been demonstrated to account for 3.2% of the variation in MFO, with carbohydrate and fat intakes contributing either negatively or positively to MFO, respectively (Fletcher et al., 2017). With astaxanthin uptake significantly enhanced if consumed alongside dietary fats (Mercke Odeberg et al., 2003, Okada, Ishikura and Maoka, 2009), it is plausible to suggest that the chronic dietary intake of each participant recruited in the current thesis may have impacted the uptake and potential efficacy of astaxanthin on each outcome variable reported. As such, chronic dietary intake should be considered as a potential confounding variable that requires greater control during future practice within this research area.

6.5. Practical Implications and Conclusion

The outcomes reported in this thesis offer an interesting insight into the potential application of astaxanthin supplementation in exercising humans. Based on the data presented in Chapter 3 and Chapter 4 recommendations for the long-term supplementation of 4 $mg \cdot day^{-1}$ or 12 $mg \cdot day^{-1}$ astaxanthin for purposes of attenuating the muscle damage response to exercise or enhancing the fat oxidative capacity during submaximal exercise are seemingly unjustified in recreationally active males. The lack of an astaxanthin effect reported could, however, be attributed to the supplementation

strategy utilised in both Chapter 3 and Chapter 4, with an ergogenic benefit of astaxanthin elicited in Chapter 5 following a shorter duration supplementation period. Indeed, the findings of Chapter 5 suggest that supplementation with 12 $mg \cdot day^{-1}$ astaxanthin for 7 days can improve performance and promote fat oxidation during endurance-type performance events. To enable the successful integration of astaxanthin supplementation in sport nutrition future investigations should aim to develop an optimal supplementation strategy for astaxanthin intake based upon human pharmacokinetic data. With this knowledge, further explorations into the efficacy of astaxanthin supplementation in exercising humans can be made while using a supplementation strategy that has been developed from scientifically rigorous, evidence-based practice.

References

ACHTEN, J., GLEESON, M., and JEUKENDRUP, A.E., 2002. Determination of the exercise intensity that elicits maximal fat oxidation. *Medicine and science in sports and exercise*. 34 (1), pp. 92–7.

ACHTEN, J., VENABLES, M.C., and JEUKENDRUP, A.E., 2003. Fat oxidation rates are higher during running compared with cycling over a wide range of intensities. *Metabolism: clinical and experimental*. 52 (6), pp. 747–52.

ALESSIO, H.M., HAGERMAN, A.E., FULKERSON, B.K., AMBROSE, J., RICE, R.E., and WILEY, R.L., 2000. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Medicine and science in sports and exercise*. 32 (9), pp. 1576–81.

AMBATI, R., MOI, P., RAVI, S., and ASWATHANARAYANA, R., 2014. Astaxanthin: Sources, Extraction, Stability, Biological Activities and Its Commercial Applications—A Review. *Marine Drugs*. 12 (1), pp. 128–152.

AOI, W., MAOKA, T., ABE, R., FUJISHITA, M., and TOMINAGA, K., 2018. Comparison of the effect of nonnesterified and esterified astaxanthins on endurance performance in mice. *J. Clin. Biochem. Nutr*. 62 (2), pp. 161–166.

AOI, W., NAITO, Y., SAKUMA, K., KUCHIDE, M., TOKUDA, H., MAOKA, T., TOYOKUNI, S., OKA, S., YASUHARA, M., and YOSHIKAWA, T., 2003. Astaxanthin Limits Exercise-Induced Skeletal and Cardiac Muscle Damage in Mice. *Antioxidants & Redox Signaling*. 5 (1), pp. 139–144.

AOI, W., NAITO, Y., TAKANAMI, Y., ISHII, T., KAWAI, Y., AKAGIRI, S., KATO, Y., OSAWA, T., and YOSHIKAWA, T., 2008. Astaxanthin improves muscle lipid metabolism in exercise via inhibitory effect of oxidative CPT I modification. *Biochemical and Biophysical Research Communications*. 366 (4), pp. 892–897.

ASTAREAL®, 2019. *Schematic depicting the antioxidant ability of astaxanthin when compared to other popular phytochemicals*.

BARALIC, I., ANDJELKOVIC, M., DJORDJEVIC, B., DIKIC, N., RADIVOJEVIC, N., SUZIN-ZIVKOVIC, V., RADOJEVIC-SKODRIC, S., and PEJIC, S., 2015. Effect of Astaxanthin Supplementation on Salivary IgA, Oxidative Stress, and Inflammation in Young Soccer Players. *Evidence-Based Complementary and Alternative Medicine*. pp. 1–9.

BAST, A. and HAENEN, G.R.M.., 2015. *Nutritional Antioxidants: It Is Time to Categorise*. Antioxidants in Sport Nutrition.

BIJUR, P.E., SILVER, W., and GALLAGHER, E.J., 2001. Reliability of the Visual Analog Scale for Measurement of Acute Pain. *Academic Emergency Medicine*. 8 (12), pp. 1153–1157.

BISHOP, D.J., BOTELLA, J., GENDERS, A.J., LEE, M.J.-C., SANER, N.J., KUANG, J., YAN, X., and GRANATA, C., 2019. High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research

Directions. *Physiology*. 34 (1), pp. 56–70.

BLOOMER, R.J., FRY, A., SCHILLING, B., CHIU, L., HORI, N., and WEISS, L., 2005. Astaxanthin supplementation does not attenuate muscle injury following eccentric exercise in resistance-trained men. *International journal of sport nutrition and exercise metabolism*. 15 (4), pp. 401–12.

BLOOMER, R.J., FRY, A.C., FALVO, M.J., and MOORE, C.A., 2007. Protein carbonyls are acutely elevated following single set anaerobic exercise in resistance trained men. *Journal of Science and Medicine in Sport*. 10 (6), pp. 411–417.

BONAVENTURA, J.M., SHARPE, K., KNIGHT, E., FULLER, K.L., TANNER, R.K., and GORE, C.J., 2015. Reliability and Accuracy of Six Hand-Held Blood Lactate Analysers. *Journal of Sports Science & Medicine*. 14 (1), p. 203.

BORG, G., LJUNGGREN, G., and CECI, R., 1985. The increase of perceived exertion, aches and pain in the legs, heart rate and blood lactate during exercise on a bicycle ergometer. *European journal of applied physiology and occupational physiology*. 54 (4), pp. 343–9.

BORG, G.A., 1982. Psychophysical bases of perceived exertion. *Medicine and science in sports and exercise*. 14 (5), pp. 377–81.

BOVERIS, A. and CHANCE, B., 1973. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *The Biochemical journal*. 134 (3), pp. 707–16.

BOWTELL, J.L., SUMNERS, D.P., DYER, A., FOX, P., and MILEVA, K.N., 2011. Montmorency cherry juice reduces muscle damage caused by intensive strength exercise. *Medicine and science in sports and exercise*. 43 (8), pp. 1544–51.

BURKE, L.M., 2015. Re-Examining High-Fat Diets for Sports Performance: Did We Call the 'Nail in the Coffin' Too Soon? *Sports Medicine*. 45 (S1), pp. 33–49.

BURKE, L.M. and PEELING, P., 2018. Methodologies for Investigating Performance Changes With Supplement Use. *International Journal of Sport Nutrition and Exercise Metabolism*. 28 (2), pp. 159–169.

BUSSO, T., 2003. Variable Dose-Response Relationship between Exercise Training and Performance. *Medicine & Science in Sports & Exercise*. 35 (7), pp. 1188–1195.

CARTER, J. and JEUKENDRUP, A., 2002. Validity and reliability of three commercially available breath-by-breath respiratory systems. *European Journal of Applied Physiology*. 86 (5), pp. 435–441.

CHOI, H.D., YOUN, Y.K., and SHIN, W.G., 2011. Positive Effects of Astaxanthin on Lipid Profiles and Oxidative Stress in Overweight Subjects. *Plant Foods for Human Nutrition*. 66 (4), pp. 363–369.

CLARK, B., CLARK, B., PATON, C.D., and O'BRIEN, B.J., 2014. *The Reliability of Performance During Computer-Simulated Varying Gradient Cycling Time Trials*. Journal of Science and Cycling. Cycling Research Center.

CLEOPHAS, T.J.M. and DE VOGEL, E.M., 1998. Crossover studies are a better format for comparing equivalent treatments than parallel-group studies. *Pharmacy World and Science*. 20 (3), pp. 113–117.

CLOSE, G.L., ASHTON, T., CABLE, T., DORAN, D., HOLLOWAY, C., MCARDLE, F., and MACLAREN, D.P.M., 2006. Ascorbic acid supplementation does not attenuate post-exercise muscle soreness following muscle-damaging exercise but may delay the recovery process. *The British journal of nutrition*. 95 (5), pp. 976–81.

CLOSE, G.L., ASHTON, T., CABLE, T., DORAN, D., and MACLAREN, D.P.M., 2004. Eccentric exercise, isokinetic muscle torque and delayed onset muscle soreness: the role of reactive oxygen species. *European Journal of Applied Physiology*. 91 (5–6), pp. 615–621.

COHEN, J., 1988. *Statistical power analysis for the behavioral sciences*. L. Erlbaum Associates.

COMMONER, B., TOWNSEND, J., and PAKE, G.E., 1954. Free radicals in biological materials. *Nature*. 174 (4432), pp. 689–91.

CONNOLLY, D.A.J., MCHUGH, M.P., PADILLA-ZAKOUR, O.I., CARLSON, L., and SAYERS, S.P., 2006. Efficacy of a tart cherry juice blend in preventing the symptoms of muscle damage * Commentary 1 * Commentary 2. *British Journal of Sports Medicine*. 40 (8), pp. 679–683.

CONNOLLY, D.A.J., SAYERS, S.P., and MCHUGH, M.P., 2003. Treatment and prevention of delayed onset muscle soreness. *Journal of strength and conditioning research*. 17 (1), pp. 197–208.

CORAL-HINOSTROZA, G.N., YTRESTØYL, T., RUYTER, B., and BJERKENG, B., 2004. Plasma appearance of unesterified astaxanthin geometrical E/Z and optical R/S isomers in men given single doses of a mixture of optical 3 and 3′R/S isomers of astaxanthin fatty acyl diesters. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 139 (1–3), pp. 99–110.

COYLE, E.F., COGGAN, A.R., HEMMERT, M.K., and IVY, J.L., 1986. Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *Journal of applied physiology (Bethesda, Md. : 1985)*. 61 (1), pp. 165–72.

CRISWELL, D., POWERS, S., DODD, S., LAWLER, J., EDWARDS, W., RENSHLER, K., and GRINTON, S., 1993. High intensity training-induced changes in skeletal muscle antioxidant enzyme activity. *Medicine and science in sports and exercise*. 25 (10), pp. 1135–40.

CROCI, I., BORRANI, F., BYRNE, N., WOOD, R., HICKMAN, I., CHENEVIÈRE, X., and MALATESTA, D., 2014. Reproducibility of Fatmax and Fat Oxidation Rates during Exercise in Recreationally Trained Males. *PLoS ONE*. 9 (6), p. e97930.

CULOTTA, V.C., YANG, M., and O'HALLORAN, T. V., 2006. Activation of superoxide dismutases: Putting the metal to the pedal. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1763 (7), pp. 747–758.

CURRELL, K. and JEUKENDRUP, A.E., 2008. Validity, Reliability and Sensitivity of Measures of Sporting Performance. *Sports Medicine*. 38 (4), pp. 297–316.

DALLE-DONNE, I., ROSSI, R., COLOMBO, R., GIUSTARINI, D., and MILZANI, A., 2006. Biomarkers of Oxidative Damage in Human Disease. *Clinical Chemistry*. 52 (4), pp. 601–623.

DAVIES, K.J., MAGUIRE, J.J., BROOKS, G.A., DALLMAN, P.R., and PACKER, L., 1982. Muscle mitochondrial bioenergetics, oxygen supply, and work capacity during dietary iron deficiency and repletion. *The American journal of physiology*. 242 (6), pp. E418-27.

DEMETRIUS, L., 2005. Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. *EMBO reports*. 6 Spec No (Suppl 1), pp. S39-44.

DJORDJEVIC, B., BARALIC, I., KOTUR-STEVULJEVIC, J., STEFANOVIC, A., IVANISEVIC, J., RADIVOJEVIC, N., ANDJELKOVIC, M., and DIKIC, N., 2012. Effect of astaxanthin supplementation on muscle damage and oxidative stress markers in elite young soccer players. *The Journal of sports medicine and physical fitness*. 52 (4), pp. 382–92.

DONE, A.J. and TRAUSTADÓTTIR, T., 2016. Nrf2 mediates redox adaptations to exercise. *Redox biology*. 10, pp. 191–199.

DONG, J., CHEN, P., WANG, R., YU, D., ZHANG, Y., and XIAO, W., 2011. NADPH oxidase: a target for the modulation of the excessive oxidase damage induced by overtraining in rat neutrophils. *International journal of biological sciences*. 7 (6), pp. 881–91.

DRÖGE, W., 2002. Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews*. 82 (1), pp. 47–95.

EARNEST, C.P., LUPO, M., WHITE, K.M., and CHURCH, T.S., 2011. Effect of astaxanthin on cycling time trial performance. *International journal of sports medicine*. 32 (11), pp. 882–8.

EFSA FEEDAP PANEL, 2005. Opinion of the Scientific Panel on additives and products or substances used in animal feed (FEEDAP) on the safety of use of colouring agents in animal nutrition - PART I. General Principles and Astaxanthin. *EFSA Journal*. 3 (12), p. 291.

EFSA FEEDAP PANEL, 2014. Scientific Opinion on the safety and efficacy of synthetic astaxanthin as feed additive for salmon and trout, other fish, ornamental fish, crustaceans and ornamental birds. *EFSA Journal*. 12 (6), p. 3724.

EFSA NDA PANEL, 2014. Scientific Opinion on the safety of astaxanthin-rich ingredients (AstaREAL A1010 and AstaREAL L10) as novel food ingredients EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *EFSA Journal*. 1212 (35).

EUSTACE, S.J., PAGE, R.M., and GREIG, M., 2018. Angle-Specific Isokinetic Metrics Highlight Strength Training Needs of Elite Youth Soccer Players. *Journal of Strength and Conditioning Research*. p. 1.

FAULKNER, J. and ESTON, R., 2007. Overall and peripheral ratings of perceived exertion during a graded exercise test to volitional exhaustion in individuals of high and low fitness. *European journal of applied physiology*. 101 (5), pp. 613–20.

FLETCHER, G., EVES, F.F., GLOVER, E.I., ROBINSON, S.L., VERNOOIJ, C.A., THOMPSON, J.L., and WALLIS, G.A., 2017. Dietary intake is independently associated with the maximal capacity for fat oxidation during exercise. *The American Journal of Clinical Nutrition*. 105 (4), pp. 864–872.

GOEDECKE, J.H., ST CLAIR GIBSON, A., GROBLER, L., COLLINS, M., NOAKES, T.D., and LAMBERT, E. V, 2000. Determinants of the variability in respiratory exchange ratio at rest and during exercise in trained athletes. *American journal of physiology. Endocrinology and metabolism*. 279 (6), pp. E1325-34.

GOMEZ-CABRERA, M.C., DOMENECH, E., ROMAGNOLI, M., ARDUINI, A., BORRAS, C., PALLARDO, F.V., SASTRE, J., and VIÑA, J., 2008. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *The American journal of clinical nutrition*. 87 (1), pp. 142–9.

GOTO, S., KOGURE, K., ABE, K., KIMATA, Y., KITAHAMA, K., YAMASHITA, E., and TERADA, H., 2001. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochimica et biophysica acta*. 1512 (2), pp. 251–8.

GRANATA, C., OLIVEIRA, R.S.F., LITTLE, J.P., RENNER, K., and BISHOP, D.J., 2016. Training intensity modulates changes in PGC-1α and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. *The FASEB Journal*. 30 (2), pp. 959–970.

GUERIN, M., HUNTLEY, M., and OLAIZOLA, M., 2003. Haematococcus astaxanthin: applications for human health and nutrition. *Trends in Biotechnology*. 21 (5), pp. 210–216.

HALLIWELL, B., 1994. Free radicals and antioxidants: a personal view. *Nutrition reviews*. 52 (8 Pt 1), pp. 253–65.

HALLIWELL, B., CLEMENT, M. V, and LONG, L.H., 2000. Hydrogen peroxide in the human body. *FEBS letters*. 486 (1), pp. 10–3.

HALLIWELL, B. and GUTTERIDGE, J., 2007. *Free Radicals in Biology and Medicine*. Fourth. Oxford: Oxford University Press.

HARRIS, C.M., SANDERS, S.A., and MASSEY, V., 1999. Role of the flavin midpoint potential and NAD binding in determining NAD versus oxygen reactivity of xanthine oxidoreductase. *The Journal of biological chemistry*. 274 (8), pp. 4561–9.

HAWLEY, J.A., BROUNS, F., and JEUKENDRUP, A., 1998. Strategies to enhance fat utilisation during exercise. *Sports medicine (Auckland, N.Z.)*. 25 (4), pp. 241–

57.

HELLSTEN, Y., FRANDSEN, U., ORTHENBLAD, N., SJØDIN, B., and RICHTER, E.A., 1997. Xanthine oxidase in human skeletal muscle following eccentric exercise: a role in inflammation. *The Journal of physiology*. pp. 239–48.

HIDALGO, C., SÁNCHEZ, G., BARRIENTOS, G., and ARACENA-PARKS, P., 2006. A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 S -glutathionylation. *The Journal of biological chemistry*. 281 (36), pp. 26473–82.

HILL, J., HOWATSON, G., VAN SOMEREN, K., LEEDER, J., and PEDLAR, C., 2014. Compression garments and recovery from exercise-induced muscle damage: a meta-analysis. *British journal of sports medicine*. 48 (18), pp. 1340–6.

HIRSCHFIELD, W., MOODY, M.R., O'BRIEN, W.E., GREGG, A.R., BRYAN, R.M., and REID, M.B., 2000. Nitric oxide release and contractile properties of skeletal muscles from mice deficient in type III NOS. *American journal of physiology. Regulatory, integrative and comparative physiology*. 278 (1), pp. R95–R100.

HOLMGREN, A., JOHANSSON, C., BERNDT, C., LÖNN, M.E., HUDEMANN, C., and LILLIG, C.H., 2005. Thiol redox control via thioredoxin and glutaredoxin systems. *Biochemical Society Transactions*. 33 (6), p. 1375.

HOWATSON, G. and VAN SOMEREN, K.A., 2007. Evidence of a contralateral repeated bout effect after maximal eccentric contractions. *European Journal of Applied Physiology*. 101 (2), pp. 207–214.

HOWATSON, G. and VAN SOMEREN, K.A., 2008. The prevention and treatment of exercise-induced muscle damage. *Sports medicine (Auckland, N.Z.)*. 38 (6), pp. 483–503.

HUSSEIN, G., SANKAWA, U., GOTO, H., MATSUMOTO, K., and WATANABE, H., 2006. Astaxanthin, a Carotenoid with Potential in Human Health and Nutrition. *Journal of Natural Products*. 69 (3), pp. 443–449.

HUTCHISON, A.T., FLIELLER, E.B., DILLON, K.J., and LEVERETT, B.D., 2016. Black Currant Nectar Reduces Muscle Damage and Inflammation Following a Bout of High-Intensity Eccentric Contractions. *Journal of Dietary Supplements*. 13 (1), pp. 1–15.

IKEUCHI, M., KOYAMA, T., TAKAHASHI, J., and YAZAWA, K., 2006. Effects of astaxanthin supplementation on exercise-induced fatigue in mice. *Biological & pharmaceutical bulletin*. 29 (10), pp. 2106–10.

JACKSON, M.J., VASILAKI, A., and MCARDLE, A., 2016. Cellular mechanisms underlying oxidative stress in human exercise. *Free Radical Biology and Medicine*. 98, pp. 13–17.

JAESCHKE, H., 2010. Antioxidant Defense Mechanisms. In: *Comprehensive Toxicology*. Elsevier. pp. 319–337.

JEUKENDRUP, A.E. and WALLIS, G.A., 2005. Measurement of Substrate Oxidation During Exercise by Means of Gas Exchange Measurements. *International Journal of Sports Medicine*. 26, pp. S28–S37.

KARPPI, J., RISSANEN, T.H., NYYSSÖNEN, K., KAIKKONEN, J., OLSSON, A.G., VOUTILAINEN, S., and SALONEN, J.T., 2007. Effects of Astaxanthin Supplementation on Lipid Peroxidation. *International Journal for Vitamin and Nutrition Research*. 77 (1), pp. 3–11.

KASPAR, J.W., NITURE, S.K., and JAISWAL, A.K., 2009. Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free radical biology & medicine*. 47 (9), pp. 1304–9.

KEHRER, J.P. and KLOTZ, L.-O., 2015. Free radicals and related reactive species as mediators of tissue injury and disease: implications for Health. *Critical Reviews in Toxicology*. 45 (9), pp. 765–798.

KERKSICK, C.M. and ZUHL, M., 2015. *Mechanisms of Oxidative Damage and Their Impact on Contracting Muscle*. Antioxidants in Sport Nutrition. CRC Press/Taylor & Francis.

KIDD, P., 2011. Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. *Alternative medicine review : a journal of clinical therapeutic*. 16 (4), pp. 355–64.

KOBZIK, L., REID, M.B., BREDT, D.S., and STAMLER, J.S., 1994. Nitric oxide in skeletal muscle. *Nature*. 372 (6506), pp. 546–548.

KOBZIK, L., STRINGER, B., BALLIGAND, J.., REID, M.B., and STAMLER, J.S., 1995. Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. *Biochemical and biophysical research communications*. 211 (2), pp. 375–81.

KUPCINSKAS, L., LAFOLIE, P., LIGNELL, Å., KIUDELIS, G., JONAITIS, L., ADAMONIS, K., ANDERSEN, L.P., and WADSTRÖM, T., 2008. Efficacy of the natural antioxidant astaxanthin in the treatment of functional dyspepsia in patients with or without Helicobacter pylori infection: A prospective, randomized, double blind, and placebo-controlled study. *Phytomedicine*. 15 (6–7), pp. 391–399.

KURASHIGE, M., OKIMASU, E., INOUE, M., and UTSUMI, K., 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiological chemistry and physics and medical NMR*. 22 (1), pp. 27–38.

LAKENS, D., 2013. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. *Frontiers in Psychology*. 4.

LANDIS, J.R. and KOCH, G.G., 1977. The Measurement of Observer Agreement for Categorical Data. *Biometrics*. 33 (1), p. 159.

LAURSEN, P.B., SHING, C.M., and JENKINS, D.G., 2003. Reproducibility of a Laboratory-Based 40-km Cycle Time-Trial on a Stationary Wind-Trainer in Highly Trained Cyclists. *International Journal of Sports Medicine*. 24 (7), pp. 481–485.

LEE, S.-J., BAI, S.-K., LEE, K.-S., NAMKOONG, S., NA, H.-J., HA, K.-S., HAN, J.-A., YIM, S.-V., CHANG, K., KWON, Y.-G., LEE, S.K., and KIM, Y.-M., 2003. Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I(kappa)B kinase-dependent NF-kappaB activation. *Molecules and cells*. 16 (1), pp. 97–105.

LEEDER, J., GISSANE, C., VAN SOMEREN, K., GREGSON, W., and HOWATSON, G., 2012. Cold water immersion and recovery from strenuous exercise: a meta-analysis. *British journal of sports medicine*. 46 (4), pp. 233–40.

LEEUWENBURGH, C., HOLLANDER, J., LEICHTWEIS, S., GRIFFITHS, M., GORE, M., and JI, L.L., 1997. Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. *The American journal of physiology*. 272 (1 Pt 2), pp. R363-9.

LIPINSKI, B., 2011. Hydroxyl radical and its scavengers in health and disease. *Oxidative medicine and cellular longevity*. 2011, p. 809696.

LIU, P.H., AOI, W., TAKAMI, M., TERAJIMA, H., TANIMURA, Y., NAITO, Y., ITOH, Y., and YOSHIKAWA, T., 2014. The astaxanthin-induced improvement in lipid metabolism during exercise is mediated by a PGC-1α increase in skeletal muscle. *Journal of Clinical Biochemistry and Nutrition*. 54 (2), pp. 86–89.

LOSCHEN, G., AZZI, A., RICHTER, C., and FLOHÉ, L., 1974. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS letters*. 42 (1), pp. 68–72.

MARGARITELIS, N.V., COBLEY, J.N., PASCHALIS, V., VESKOUKIS, A.S., THEODOROU, A.A., KYPAROS, A., and NIKOLAIDIS, M.G., 2016. Going retro: Oxidative stress biomarkers in modern redox biology. *Free Radical Biology and Medicine*. 98, pp. 2–12.

MARKLUND, S., 1976. Spectrophotometric Study of Spontaneous Disproportionation of Superoxide Anion Radical and Sensitive Direct Assay for Superoxide Dismutase*. *THE JOURNAL OF BIOLOGICAL CHEMISTRY*. 251 (23), pp. 7504–7507.

MCCORD, J.M. and FRIDOVICH, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *The Journal of biological chemistry*. 244 (22), pp. 6049–55.

MCHUGH, M.P., 2003. Recent advances in the understanding of the repeated bout effect: the protective effect against muscle damage from a single bout of eccentric exercise. *Scandinavian journal of medicine & science in sports*. 13 (2), pp. 88–97.

MCHUGH, M.P., CONNOLLY, D.A., ESTON, R.G., and GLEIM, G.W., 1999. Exercise-induced muscle damage and potential mechanisms for the repeated bout effect. *Sports medicine (Auckland, N.Z.)*. 27 (3), pp. 157–70.

MCLEAY, Y., BARNES, M.J., MUNDEL, T., HURST, S.M., HURST, R.D., and STANNARD, S.R., 2012. Effect of New Zealand blueberry consumption on recovery from eccentric exercise-induced muscle damage. *Journal of the*

International Society of Sports Nutrition. 9.

DI MEO, S. and VENDITTI, P., 2001. Mitochondria in exercise-induced oxidative stress. *Biological signals and receptors*. 10 (1–2), pp. 125–40.

MERCKE ODEBERG, J., LIGNELL, A., PETTERSSON, A., and HÖGLUND, P., 2003. Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences*. 19 (4), pp. 299–304.

MICKLEWRIGHT, D., ST CLAIR GIBSON, A., GLADWELL, V., and AL SALMAN, A., 2017. Development and Validity of the Rating-of-Fatigue Scale. *Sports Medicine*. 47 (11), pp. 2375–2393.

MIKI, W., 1991. Biological functions and activities of animal carotenoids. *Pure and Applied Chemistry*. 63 (1), pp. 141–146.

MINDERICO, C.S., SILVA, A.M., TEIXEIRA, P.J., SARDINHA, L.B., HULL, H.R., and FIELDS, D.A., 2006. Validity of air-displacement plethysmography in the assessment of body composition changes in a 16-month weight loss program. *Nutrition & Metabolism*. 3 (1), p. 32.

NAGUIB, Y.M., 2000. Antioxidant activities of astaxanthin and related carotenoids. *Journal of agricultural and food chemistry*. 48 (4), pp. 1150–4.

NEUBAUER, O., KÖNIG, D., KERN, N., NICS, L., and WAGNER, K.-H., 2008. No Indications of Persistent Oxidative Stress in Response to an Ironman Triathlon. *Medicine & Science in Sports & Exercise*. 40 (12), pp. 2119–2128.

NEUBAUER, O. and YFANTI, C., 2015. *Antioxidants in Athlete's Basic Nutrition: Considerations towards a Guideline for the Intake of Vitamin C and Vitamin E*. Antioxidants in Sport Nutrition. CRC Press/Taylor & Francis.

OHKUWA, T., SATO, Y., and NAOI, M., 1997. Glutathione status and reactive oxygen generation in tissues of young and old exercised rats. *Acta physiologica Scandinavica*. 159 (3), pp. 237–44.

OKADA, Y., ISHIKURA, M., and MAOKA, T., 2009. Bioavailability of Astaxanthin in Haematococcus Algal Extract: The Effects of Timing of Diet and Smoking Habits. *Bioscience, Biotechnology, and Biochemistry*. 73 (9), pp. 1928–1932.

ØSTERLIE, M., BJERKENG, B., and LIAAEN-JENSEN, S., 2000. Plasma appearance and distribution of astaxanthin E/Z and R/S isomers in plasma lipoproteins of men after single dose administration of astaxanthin. *The Journal of nutritional biochemistry*. 11 (10), pp. 482–90.

PACHER, P., BECKMAN, J.S., and LIAUDET, L., 2007. Nitric Oxide and Peroxynitrite in Health and Disease. *Physiological Reviews*. 87 (1), pp. 315–424.

PALMER, G., DENNIS, S., NOAKES, T., and HAWLEY, J., 1996. Assessment of the Reproducibility of Performance Testing on an Air-Braked Cycle Ergometer. *International Journal of Sports Medicine*. 17 (04), pp. 293–298.

PALOZZA, P. and KRINSKY, N.I., 1992. Astaxanthin and canthaxanthin are potent

antioxidants in a membrane model. *Archives of biochemistry and biophysics*. 297 (2), pp. 291–5.

DE PAUW, K., ROELANDS, B., CHEUNG, S.S., DE GEUS, B., RIETJENS, G., and MEEUSEN, R., 2013. *Guidelines to Classify Subject Groups in Sport-Science Research*. International Journal of Sports Physiology and Performance.

PERLMAN, R.L., 2016. Mouse models of human disease: An evolutionary perspective. *Evolution, medicine, and public health*. 2016 (1), pp. 170–6.

PETERNELJ, T.T. and COOMBES, J.S., 2011. Antioxidant supplementation during exercise training: Beneficial or detrimental? *Sports Medicine*. 41 (12), pp. 1043–1069.

PINGITORE, A., LIMA, G.P.P., MASTORCI, F., QUINONES, A., IERVASI, G., and VASSALLE, C., 2015. Exercise and oxidative stress: Potential effects of antioxidant dietary strategies in sports. *Nutrition*. 31 (7–8), pp. 916–922.

PODEROSO, J.J., CARRERAS, M.C., LISDERO, C., RIOBÓ, N., SCHÖPFER, F., and BOVERIS, A., 1996. Nitric Oxide Inhibits Electron Transfer and Increases Superoxide Radical Production in Rat Heart Mitochondria and Submitochondrial Particles. *Archives of Biochemistry and Biophysics*. 328 (1), pp. 85–92.

POWERS, S.K., CRISWELL, D., LAWLER, J., JI, L.L., MARTIN, D., HERB, R.A., and DUDLEY, G., 1994. Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. *The American journal of physiology*. 266 (2 Pt 2), pp. R375-80.

POWERS, S.K., DERUISSEAU, K.C., QUINDRY, J., and HAMILTON, K.L., 2004. Dietary antioxidants and exercise. *Journal of Sports Sciences*. 22 (1), pp. 81–94.

POWERS, S.K., DUARTE, J., KAVAZIS, A.N., and TALBERT, E.E., 2010. Reactive oxygen species are signalling molecules for skeletal muscle adaptation. *Experimental Physiology*. 95 (1), pp. 1–9.

POWERS, S.K. and JACKSON, M.J., 2008. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiological reviews*. 88 (4), pp. 1243–76.

POWERS, S.K., JI, L.L., KAVAZIS, A.N., and JACKSON, M.J., 2011. Reactive Oxygen Species: Impact on Skeletal Muscle. In: *Comprehensive Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc.

POWERS, S.K., NELSON, W.B., and HUDSON, M.B., 2011. Exercise-induced oxidative stress in humans: Cause and consequences. *Free Radical Biology and Medicine*. 51 (5), pp. 942–950.

POWERS, S.K., TALBERT, E.E., and ADHIHETTY, P.J., 2011. Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. *The Journal of Physiology*. 589 (9), pp. 2129–2138.

PROSKE, U. and ALLEN, T.J., 2005. Damage to skeletal muscle from eccentric exercise. *Exercise and sport sciences reviews*. 33 (2), pp. 98–104.

PROSKE, U. and MORGAN, D.L., 2001. Muscle damage from eccentric exercise:

mechanism, mechanical signs, adaptation and clinical applications. *The Journal of physiology*. 537 (Pt 2), pp. 333–45.

RADAK, Z., ISHIHARA, K., TEKUS, E., VARGA, C., POSA, A., BALOGH, L., BOLDOGH, I., and KOLTAI, E., 2017. Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. *Redox Biology*. 12, pp. 285–290.

RADAK, Z., ZHAO, Z., KOLTAI, E., OHNO, H., and ATALAY, M., 2013. Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. *Antioxidants & redox signaling*. 18 (10), pp. 1208–46.

RAJADHYAKSHA, A., RODRIGUEZ, M., and NICHOLS, J.H., 2007. Evaluation of the HemoCue Glucose 201 Room-Temperature Microcuvettes. *Point of Care: The Journal of Near-Patient Testing & Technology*. 6 (3), pp. 170–173.

RANDELL, R.K., ROLLO, I., ROBERTS, T.J., DALRYMPLE, K.J., JEUKENDRUP, A.E., CARTER, J.M., RANDELL, A., ROLLO, I., ROBERTS, T.J., DALRYMPLE, K.J., JEUKENDRUP, A.E., and CARTER, J.M., 2017. Maximal Fat Oxidation Rates in an Athletic Population. *Med. Sci. Sports Exerc*. 49 (1), pp. 133–140.

REID, M., 2016a. Redox interventions to increase exercise performance. *The Journal of physiology*. 594 (18), pp. 5125–33.

REID, M., 2016b. Reactive Oxygen Species as Agents of Fatigue. *Medicine & Science in Sports & Exercise*. 48 (11), pp. 2239–2246.

REILLY, T., 1990. Human circadian rhythms and exercise. *Critical reviews in biomedical engineering*. 18 (3), pp. 165–80.

RES, P.T., CERMAK, N.M., STINKENS, R., TOLLAKSON, T.J., HAENEN, G.R., BAST, A., and VAN LOON, L.J.C., 2013. Astaxanthin Supplementation Does Not Augment Fat Use or Improve Endurance Performance. *Medicine & Science in Sports & Exercise*. 45 (6), pp. 1158–1165.

RIOBÓ, N.A., CLEMENTI, E., MELANI, M., BOVERIS, A., CADENAS, E., MONCADA, S., and PODEROSO, J.J., 2001. Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *The Biochemical journal*. 359 (Pt 1), pp. 139–45.

RISTOW, M., ZARSE, K., OBERBACH, A., KLOTING, N., BIRRINGER, M., KIEHNTOPF, M., STUMVOLL, M., KAHN, C.R., and BLUHER, M., 2009. Antioxidants prevent health-promoting effects of physical exercise in humans. *Proceedings of the National Academy of Sciences*. 106 (21), pp. 8665–8670.

ROBERTS, L.A., NOSAKA, K., COOMBES, J.S., and PEAKE, J.M., 2014. Cold water immersion enhances recovery of submaximal muscle function after resistance exercise. *AJP: Regulatory, Integrative and Comparative Physiology*. 307 (8), pp. R998–R1008.

ROBINSON, S.L., HATTERSLEY, J., FROST, G.S., CHAMBERS, E.S., and WALLIS, G.A., 2015. Maximal fat oxidation during exercise is positively

associated with 24-hour fat oxidation and insulin sensitivity in young, healthy men. *Journal of Applied Physiology*. 118 (11), pp. 1415–1422.

ROSENBERG, K., DURNIN, J.V.G.A., BARNES, E.W., COOKE, N.J., KING, A.J., PASSMORE, R., GARROW, J.S., HAWES, S.F., LE BRETON, P.E., PERMAN, E.S., PITTET, P., GYGAX, P.H., JÉQUIER, E., STOCK, M.J., and STUART, J.A., 1978. The effect of alcohol on resting metabolic rate. *British Journal of Nutrition*. 40 (02), p. 293.

RÜFER, C.E., MOESENEDER, J., BRIVIBA, K., RECHKEMMER, G., and BUB, A., 2008. Bioavailability of astaxanthin stereoisomers from wild (Oncorhynchus spp.) and aquacultured (Salmo salar) salmon in healthy men: a randomised, double-blind study. *British Journal of Nutrition*. 99 (05), pp. 1048–54.

SAHA, N., 2018. Clinical Pharmacokinetics and Drug Interactions. *Pharmaceutical Medicine and Translational Clinical Research*. pp. 81–106.

SAKELLARIOU, G.K., VASILAKI, A., PALOMERO, J., KAYANI, A., ZIBRIK, L., MCARDLE, A., and JACKSON, M.J., 2013. Studies of Mitochondrial and Nonmitochondrial Sources Implicate Nicotinamide Adenine Dinucleotide Phosphate Oxidase(s) in the Increased Skeletal Muscle Superoxide Generation That Occurs During Contractile Activity. *Antioxidants & Redox Signaling*. 18 (6), pp. 603–621.

SEN, C.K., MARIN, E., KRETZSCHMAR, M., and HÄNNINEN, O., 1992. Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *Journal of applied physiology (Bethesda, Md. : 1985)*. 73 (4), pp. 1265–72.

SHAH, M.M.R., LIANG, Y., CHENG, J.J., and DAROCH, M., 2016. Astaxanthin-Producing Green Microalga Haematococcus pluvialis: From Single Cell to High Value Commercial Products. *Frontiers in plant science*. 7, p. 531.

SHANKS, N., GREEK, R., and GREEK, J., 2009. Are animal models predictive for humans? *Philosophy, ethics, and humanities in medicine : PEHM*. 4, p. 2.

SHIMIDZU, N., GOTO, M., and MIKI, W., 1996. Carotenoids as Singlet Oxygen Quenchers in Marine Organisms. *Fisheries science*. 62 (1), pp. 134–137.

SLATTERY, K., BENTLEY, D., and COUTTS, A.J., 2015. The Role of Oxidative, Inflammatory and Neuroendocrinological Systems During Exercise Stress in Athletes: Implications of Antioxidant Supplementation on Physiological Adaptation During Intensified Physical Training. *Sports Medicine*. 45 (4), pp. 453–471.

SPILLER, G.A. and DEWELL, A., 2003. Safety of an Astaxanthin-Rich *Haematococcus pluvialis* Algal Extract: A Randomized Clinical Trial. *Journal of Medicinal Food*. 6 (1), pp. 51–56.

ST-PIERRE, J., BUCKINGHAM, J.A., ROEBUCK, S.J., and BRAND, M.D., 2002. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *The Journal of biological chemistry*. 277 (47), pp. 44784–90.

STAHL, W. and SIES, H., 2003. Antioxidant activity of carotenoids. *Molecular Aspects of Medicine*. 24 (6), pp. 345–351.

STATLAND, B.E., 1990. A review of the analytic performance of the Reflotron System for cholesterol testing. *Clinical therapeutics*. 12 (3), pp. 281–6.

STISEN, A.B., STOUGAARD, O., LANGFORT, J., HELGE, J.W., SAHLIN, K., and MADSEN, K., 2006. Maximal fat oxidation rates in endurance trained and untrained women. *European Journal of Applied Physiology*. 98 (5), pp. 497–506.

THOMSON, D.M. and WINDER, W.W., 2009. AMP-activated protein kinase control of fat metabolism in skeletal muscle. *Acta physiologica (Oxford, England)*. 196 (1), pp. 147–54.

TSEH, W., CAPUTO, J.L., and KEEFER, D.J., 2010. Validity and Reliability of the BOD POD ® S/T Tracking System. *International Journal of Sports Medicine*. 31 (10), pp. 704–708.

TSUTSUI, H., KINUGAWA, S., and MATSUSHIMA, S., 2011. Oxidative stress and heart failure. *AJP: Heart and Circulatory Physiology*. 301 (6), pp. H2181–H2190.

UTTARA, B., SINGH, A. V, ZAMBONI, P., and MAHAJAN, R.T., 2009. Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. *Current Neuropharmacology*. 7, pp. 65–74.

VENABLES, M.C., ACHTEN, J., and JEUKENDRUP, A.E., 2005. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *Journal of applied physiology (Bethesda, Md. : 1985)*. 98 (1), pp. 160–7.

VILLANUEVA, C. and KROSS, R.D., 2012. Antioxidant-Induced Stress. *Int. J. Mol. Sci*. 13, pp. 2091–2109.

VISIOLI, F. and ARTARIA, C., 2017. Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps. *Food Funct.* 8 (1), pp. 39–63.

WAGNER, K.-H., 2015. *Antioxidants in Sport Nutrition: All the Same Effectiveness?* Antioxidants in Sport Nutrition.

WARREN, G.L., LOWE, D.A., and ARMSTRONG, R.B., 1999. Measurement tools used in the study of eccentric contraction-induced injury. *Sports medicine (Auckland, N.Z.)*. 27 (1), pp. 43–59.

WEISBERG, S., 2014. *Applied linear regression*. 4th ed. New York: Wiley.

WESTERTERP-PLANTENGA, M., DIEPVENS, K., JOOSEN, A.M.C.P., BÉRUBÉ-PARENT, S., and TREMBLAY, A., 2006. Metabolic effects of spices, teas, and caffeine. *Physiology & Behavior*. 89 (1), pp. 85–91.

WOLF, A.M., ASOH, S., HIRANUMA, H., OHSAWA, I., IIO, K., SATOU, A., ISHIKURA, M., and OHTA, S., 2010. Astaxanthin protects mitochondrial redox state and functional integrity against oxidative stress. *The Journal of Nutritional Biochemistry*. 21 (5), pp. 381–389.

XU, J., RONG, S., GAO, H., CHEN, C., YANG, W., DENG, Q., HUANG, Q., XIAO,

L., and HUANG, F., 2017. A Combination of Flaxseed Oil and Astaxanthin Improves Hepatic Lipid Accumulation and Reduces Oxidative Stress in High Fat-Diet Fed Rats. *Nutrients*. 9 (3).

YANG, Y., PHAM, T.X., WEGNER, C.J., KIM, B., KU, C.S., PARK, Y.-K., and LEE, J.-Y., 2014. Astaxanthin lowers plasma TAG concentrations and increases hepatic antioxidant gene expression in diet-induced obesity mice. *British Journal of Nutrition*. 112 (11), pp. 1797–1804.

YANG, Y., SEO, J.M., NGUYEN, A., PHAM, T.X., PARK, H.J., PARK, Y., KIM, B., BRUNO, R.S., and LEE, J., 2011. Astaxanthin-rich extract from the green alga Haematococcus pluvialis lowers plasma lipid concentrations and enhances antioxidant defense in apolipoprotein E knockout mice. *The Journal of nutrition*. 141 (9), pp. 1611–7.

YEO, W.K., CAREY, A.L., BURKE, L., SPRIET, L.L., and HAWLEY, J.A., 2011. Fat adaptation in well-trained athletes: effects on cell metabolism. *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme*. 36 (1), pp. 12–22.

YU, B.P., 1994. Cellular defenses against damage from reactive oxygen species. *Physiological reviews*. 74 (1), pp. 139–62.

VAN DER ZWAARD, S., DE RUITER, C.J., NOORDHOF, D.A., STERRENBURG, R., BLOEMERS, F.W., DE KONING, J.J., JASPERS, R.T., and VAN DER LAARSE, W.J., 2016. Maximal oxygen uptake is proportional to muscle fiber oxidative capacity, from chronic heart failure patients to professional cyclists. *Journal of Applied Physiology*. 121 (3), pp. 636–645.